AD_____

Award Number: DAMD17-02-1-0349

TITLE: Identification of Novel Genes Affected by Gamma Irradiation Using a Gene-Trapped Library of Human Mammary Epithelial Cells

PRINCIPAL INVESTIGATOR: Jennifer L. Malone
Doctor Robert Ullrich

CONTRACTING ORGANIZATION: Colorado State University
Fort Collins, Colorado 80523-2002

REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Identification of Novel Genes Affected by Gamma Irradiation Using a Gene-Trapped Library of Human Mammary Epithelial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0349

6. AUTHOR(S)
Jennifer Malone
Doctor Robert Ullrich

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Colorado State University
Fort Collins, Colorado 80523-2002

E-Mail: malones41500@hotmail.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We propose that the expression of several unknown genes is affected by gamma radiation. Abnormal expression of these genes may be one of the steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contains a single mutation by gene trapping. We will be able to detect changes in the expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends procedure and sequenced. Cells that are affected by radiation will be isolated and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in ethe early stages of breast cancer progression.
The 3'RACE protocol has recently been completed and thirty one genes potential genes were sequenced. Of these, six candidate genes were found. The include: human creatine kinase gene, human androgen receptor, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF1B2), human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20. These genes will be further analyzed for their transformation properties of human mammary epithelial cells as discussed in the statement of work.

14. SUBJECT TERMS
Gamma irradiation, gene trapping, transformation

15. NUMBER OF PAGES
47

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

We propose that the expression of several unknown genes is affected by gamma irradiation. The subject and purpose of our research is that the abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single mutation by gene trapping. We will be able to detect changes in the expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression.

BODY

RESEARCH TRAINING

Ongoing training is very important throughout my predoctoral period. My department, Environmental and Radiological Health Sciences, places an important focus on training. Weekly, I attend Advanced Radiation Biology journal meetings where faculty and students interact and discuss current and relevant papers in breast cancer research and radiation effects. Each attendee presents one journal article every semester and leads the discussion. Weekly there is a Cell and Molecular Biology seminar where invited visiting speakers give a 50 minute presentation and discussion about relevant topics such as breast cancer research, cell signaling, and many more. There are also two departmental seminars I attend weekly where visiting speakers, as well as graduate students present their research. It is very important for my training that I keep current with the latest research techniques and discoveries by attending these meetings. My mentor, Dr. Robert Ullrich, is currently the Oncology Chair of the Veterinary Teaching Hospital here on campus, so I am also exposed to more clinical cancer research seminars and meetings that I attend there as well.

In October 2003 I attended the American Association for Cancer Research special conference on the Advances in Breast Cancer Research meeting in Huntington Beach, California. In February 2004 I attend the American Association for Cancer Research special conference on Radiation Biology and Cancer meeting in Dana Point, California. These meetings contributed a great deal to my overall predoctoral training by exposing me to breast cancer research scientists from all over the world. I was able to attend numerous oral and poster presentations and learn about the latest advances being made in breast cancer and radiation research.

RESEARCH PURPOSE & GOALS

We plan to identify novel genes affected by gamma irradiation and to characterize their function using a gene-trapped library of human mammary epithelial cells. We hypothesize that the mutation of these novel genes or its abnormal expression is one of the causes of early breast carcinogenesis. Mounting evidence suggests that gene products may function differently depending on cell type, developmental stage, or species. Thus, to identify novel gene(s) critical for the initiation of breast cancer, we need to study the irradiation effects of "loss of function" of a gene product in human breast epithelial cells.

The issue of how low dose gamma radiation may lead to breast cancer will be addressed by studying the genes affected by low dose gamma irradiation. We will focus on the trapped genes whose expression are immediately changed by a single dose of gamma irradiation,

determine if this is a dose-dependent effect and further analyze whether this effect can lead to transformation of the breast cells.

The following are specific aims as outlined in the approved statement of work:

Specific Aim 1: To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones.
Specific Aim 2: To determine the effect of gamma irradiation on expression of reporter protein GFP (green fluorescent protein).
Specific Aim 3: To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells.
Specific Aim 4: To identify the trapped genes affected by gamma irradiation.

RESEARCH PROGRESS

Currently, specific aim 1, specific aim 2, and specific aim 4 are completed. Specific Aim 3 is still in progress. At this point no transformation has been observed yet. Attached in the appendices are color representations of the completed construction of the gene-trapped MCF10A clonal library as seen under a fluorescent microscope. This is included in one of my PowerPoint presentations. It is clearly observed in the pictorials, that the bright green fluorescence luminating from the cells is due to the retrovirus pRET being incorporated into the genome.

A total of 192 gene-trapped clones were analyzed by the construction of a single cell assay in 96-well plates. This was done to obtain single cell clones, hopefully each representing a different trapped gene. One 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP positive pool and the other 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP negative pool. Graphical representations of the flow cytometry data are included in one of my power point presentations. Replica plating was then done from both of the original single cell assay plates for the following GFP expression levels to be measured at: basal, control, master, store at -80°C, 0.5 Gy, and 2.0 Gy gamma irradiated. GFP measurements were made with a microplate reader by the way of a sandwich ELISA assay. The sandwich ELISA assay was accomplished by first expanding the 96-well plates with the single cell clones into 24-well plates. These 24-well plates were then expanded further to allow for 2 wells for each single cell assay clone. This was done so that one well could be further expanded and frozen for later use and the other well would be utilized to collect the cell lysate from for the ELISA assay. All of the 24-well plates were then irradiated with 2.0 Gy from a ^{137}Cs source. The following antibodies were used for the sandwich ELISA assay: anti-GFP (Mouse) was the primary antibody and peroxidase IgG mouse (Rabbit) was the secondary antibody.

Graphical representations of the gene expression of GFP after 2.0 Gy gamma radiation dose from a ^{137}Cs source is attached as well. Here, clones that were up- or down-regulated at least 2-fold from basal readings were expanded for further analysis. The basal GFP readings of the gene-trapped clones are included in the appendices for comparison to the 2.0 Gy GFP readings. Out of the 192 clones analyzed, 92 were up- or down-regulated at least 2-fold in comparison to basal GFP readings. These clones were expanded in culture and RNA was collected for gene analysis.

There was a slight change in the order of the approved statement of work next. Specific aim 4 was undertaken prior to the characterization of the effect of gamma irradiation on the transformation of the MCF10A cells. This was felt to be an important substitution due to the fact

that the gene that had been trapped should be identified before transformation assays were undertaken. Transformation assays are very tedious and time consuming. If, for example, the gene trapped was an artifact, then the process of analyzing for transformation could be skipped.

Specific aim 4 dealt with the characterization of the trapped genes that were causing either an up- or down-regulation upon treatment with 2.0 Gy. To analyze this, the gene-trapped clones were expanded and RNA was collected by using Qiagen's RNeasy kit. The protocol for this procedure is attached in the appendices. The RNA was then reverse transcribed into cDNA and amplified by the use of the Advantage-GC cDNA polymerase kit from BD Biosciences and the 3'RACE protocol from Invitrogen. Both protocols are included in the appendices. Gene specific primers for the neomycin marker found on our pRET retrovirus and against the polyA tail of the endogenous gene were designed. After each step, reverse transcription, first strand cDNA synthesis, and second strand cDNA synthesis, agarose gels were run to verify that the gene products were of the correct size.

When a gene product was of the correct size they were PCR purified by Qiagen's PCR purification kit and transformed into One Shot competent E. coli cells via a TOPO Cloning kit from Invitrogen. The transformed clones were then added to LB media and grown overnight. Clones were growth had occurred were then subjected to Qiagen's mini prep kit to harvest the DNA. Protocols for both of these procedures can be found in the appendices. The mini prep clones are then subjected to PCR with M13 primers and run on 1.5% agarose gels. Gel electrophoresis images are provided in the appendices in one of my PowerPoint presentations to illustrate which clones were selected to be sequenced.

Sequencing of the positive mini prep clones was completed at Davis Sequencing which is located at the University of California at Davis. A total of 31 clones were sent off for sequencing and six yielded positive results. The six genes were determined by plugging the sequences of my clones into BLAST and searching for homologous genes. The other clones were determined to be artifacts of the cloning vector. The genes that were trapped were: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF1B2), human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.

The genes identified through sequencing analysis were expanded for RNA collection and analyzed by real-time PCR. This experiment was performed in order to analyze gene expression of the genes identified through trapping in both the gene-trapped clones and in the parental MCF10A cell line with and without ionizing radiation treatment. We felt that it was important to analyze the identified gene expression levels following IR treatment in the parental cell line to verify that in fact we were in deed seeing a radiation response. These expression levels could also then be compared to the breast cancer cell line, MCF7. In the appendices I have included my real-time PCR protocol and the sequences for the primers and probes that were utilized. Real-time PCR experiments were conducted on an Applied Biosystems 7000 Sequence Detection System with the TaqMan Gold RT-PCR Kit. Also, graphical representations of the relative gene expression of the genes of interest in various time course experiments following ionizing radiation (IR) and after varying doses of IR are included. The time course experiments were conducted at 2, 4, 8, 12, 24, and 30 hours post ionizing radiation treatment with a dose of 2.0 Gy. I am currently in the process of completing time course studies for 0.5 Gy, 1.0 Gy, and 4.0 Gy which will not be done in time to submit for this annual report. All five genes illustrated a radiation response and their relative gene expression and n-fold difference in comparison to the

parental, MCF10A cell line, were analyzed. The experimental results from the above mentioned items are all included in the appendices.

KEY RESEARCH ACCOMPLISHMENTS

- Five radiation response genes were found to be homologous to known genes through a BLAST search. These genes include: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2, human androgen receptor, human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.
- Genes of interest were found to response to a 2.0 Gy dose of ionizing radiation and time course experiments were completed to find when peak expression levels following IR occurred. This was analyzed by real-time PCR.
- Cell cycle analysis was done to verify that there was not a cell cycle delay or block causing some of the large changes in expression of some of the genes that was seen.
- Real-time PCR analysis was performed to analyze the expression of our genes of interest at 0.5 Gy, 1.0 Gy, 2.0 Gy, and 4.0 Gy doses of IR.
- One of the genes of interest, DREV1, has a small gene called DORA located in intron 4 on the complement strand. Real-time PCR analysis has been completed to investigate if its gene expression is also affected by the IR doses.

REPORTABLE OUTCOMES

- The development of a gene-trapped cell library of MCF10A cells was accomplished with the retrovirus pRET.
- On March 18, 2004 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences. The PowerPoint slides from my presentation are given in the appendices.
- I was invited to give a poster presentation at Colorado State University for the Cell and Molecular Biology Interdisciplinary Graduate Program Graduate Student and Post Doc Poster Competition on February 27, 2004. My poster abstract is found in the appendices.
- I was invited to give a poster presentation at the American Association for Cancer Research Special Conference: Radiation Biology and Cancer. It was held from February 18th through February 22nd in Dana Point, California. My abstract can be found in the appendices.
- I was invited to give a poster presentation at the American Association for Cancer Research Special Conference: Advances in Breast Cancer Research. It was held in October 2003 in Huntington Beach, California. My abstract can be found in the appendices.
- On October 23, 2003 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences. The department has doctoral students give oral presentations every semester on how their research is progressing and any new findings. The PowerPoint slides from my presentation are given in the appendices.

APPENDICES

QIAGEN RNeasy Protocol Website:
http://www1.qiagen.com/literature/handbooks/PDF/RNAStabilizationAndPurification/FromAnimalAndPlantTissuesBacteriaYeastAndFungi/RNY_Mini/1016272HBRNY_062001WW.pdf

BD Biosciences Advantage GC cDNA PCR kit manual
http://www.bdbiosciences.com/clontech/techinfo/manuals/PDF/PT1580-1.pdf

Invitrogen 3'RACE system for amplification of cDNA ends manual
https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&sku=&productDescription=102&

Invitrogen TOPO Cloning Kit manual
http://www.invitrogen.com/content/sfs/brochures/710_021849%20_B_TOPOCloning_bro.pdf

QIAGEN PCR purification kit manual
http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/QQ_Spin/1021422_HBQQSpin_072002WW.pdf

Applied Biosystems TaqMan Gold RT-PCR Kit
http://home.appliedbiosystems.com/ search product literature for manual

Reverse Transcription conditions:
25°C, 10 minutes
48°C, 30 minutes
95°C, 5 minutes

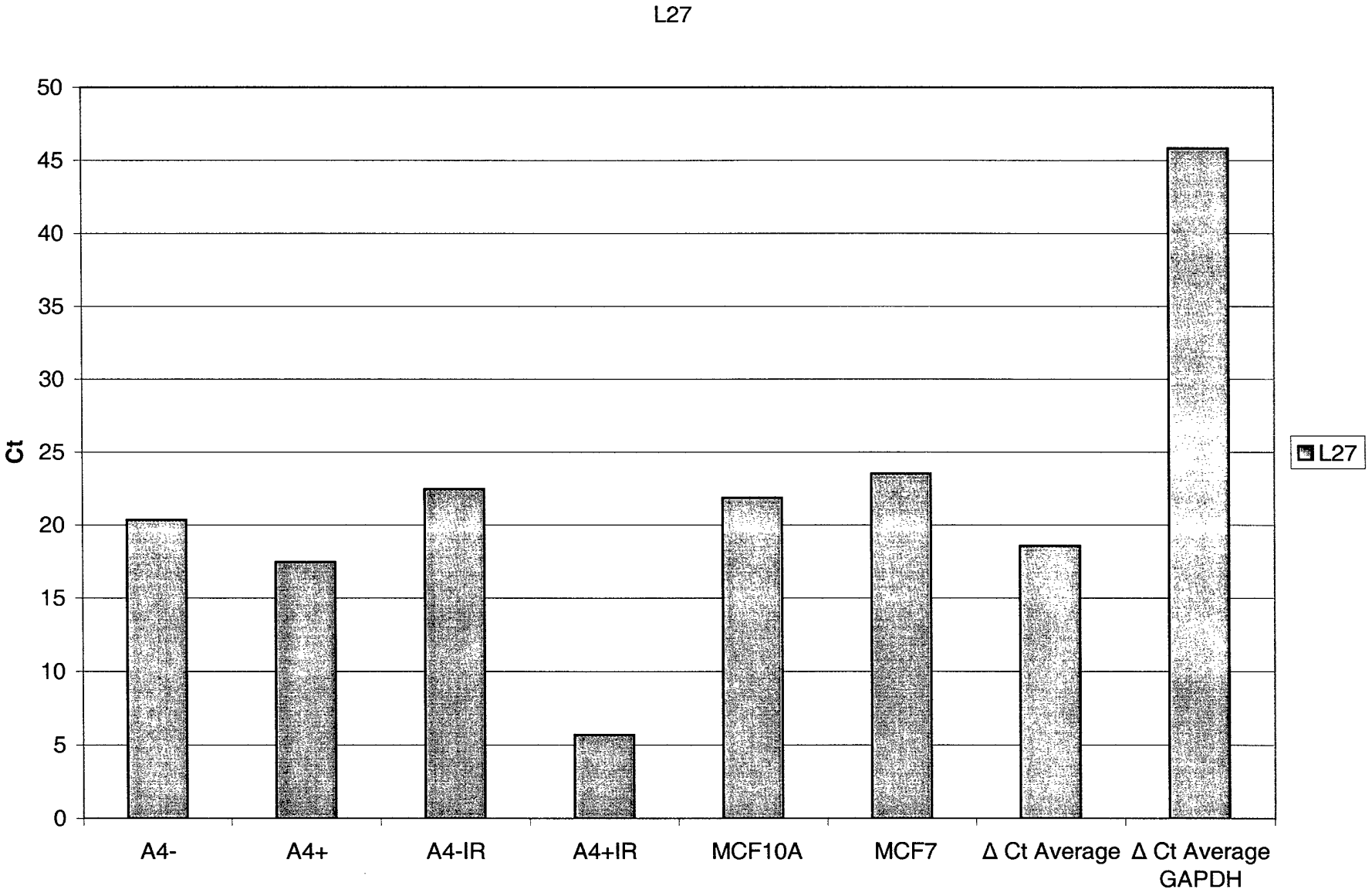
Real-Time PCR conditions
50°C, 2 minutes, 1 cycle
95°C, 10 minutes, 1 cycle
95°C, 15 seconds; 60°C, 1 minute, 40 cycles

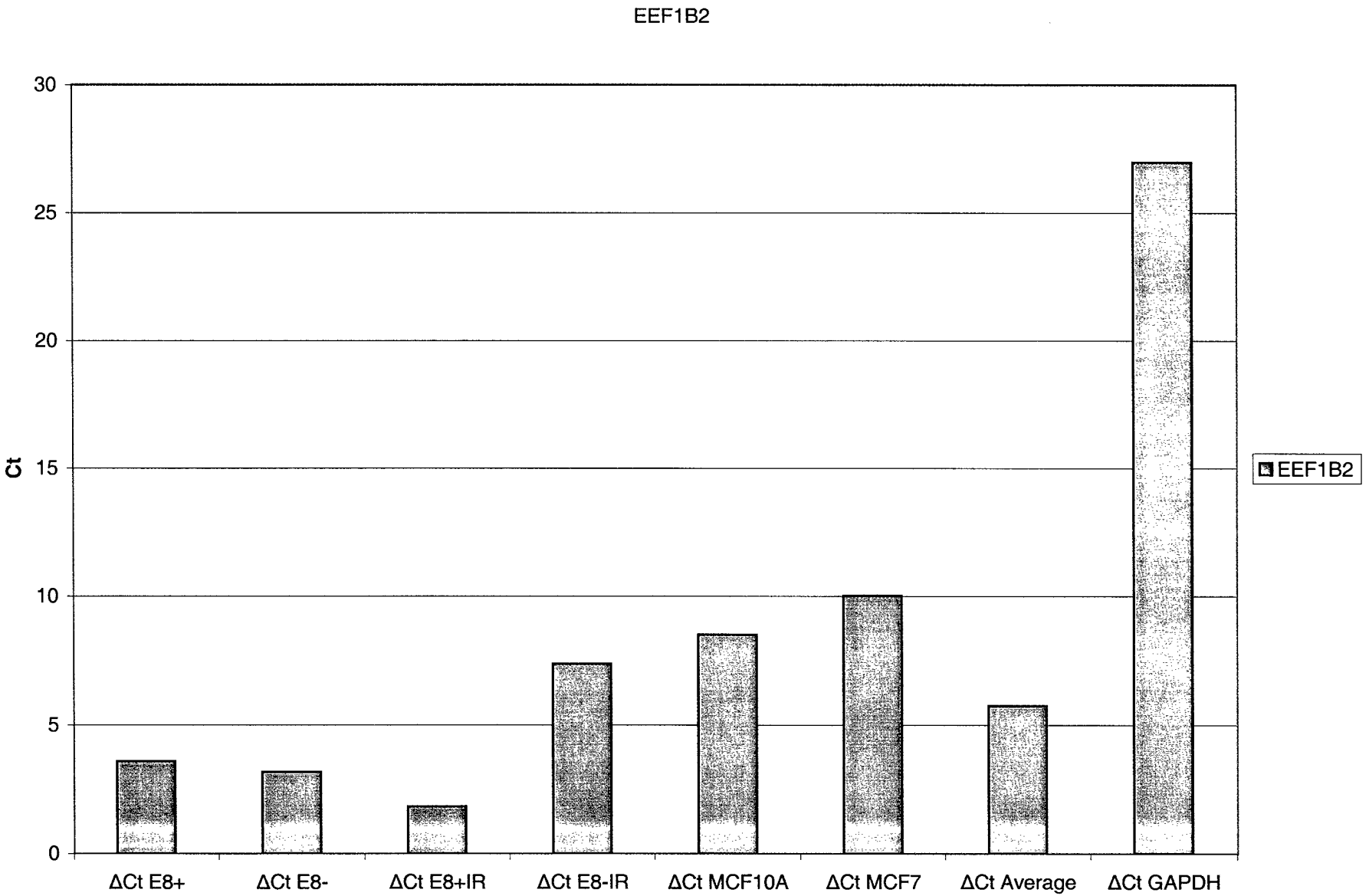
Real-Time PCR primers (concentrations in reaction at 200 nM)
Igsf6F1
ACCTTCTCCGCAACCGG
Igsf6F2
TACCTTCTCCGCAACCGG
Igsf6F3
GTACCTTCTCCGCAACCGG
Igsf6R1
GCACCGTAGCGAAACCACA
AndrogenF1
CCCTGGCGGCATGGT
AndrogenF2
ACCCTGGCGGCATGGT
AndrogenF3
TACCCTGGCGGCATGGT
AndrogenR1
CCCATTTCGCTTTTGACACA

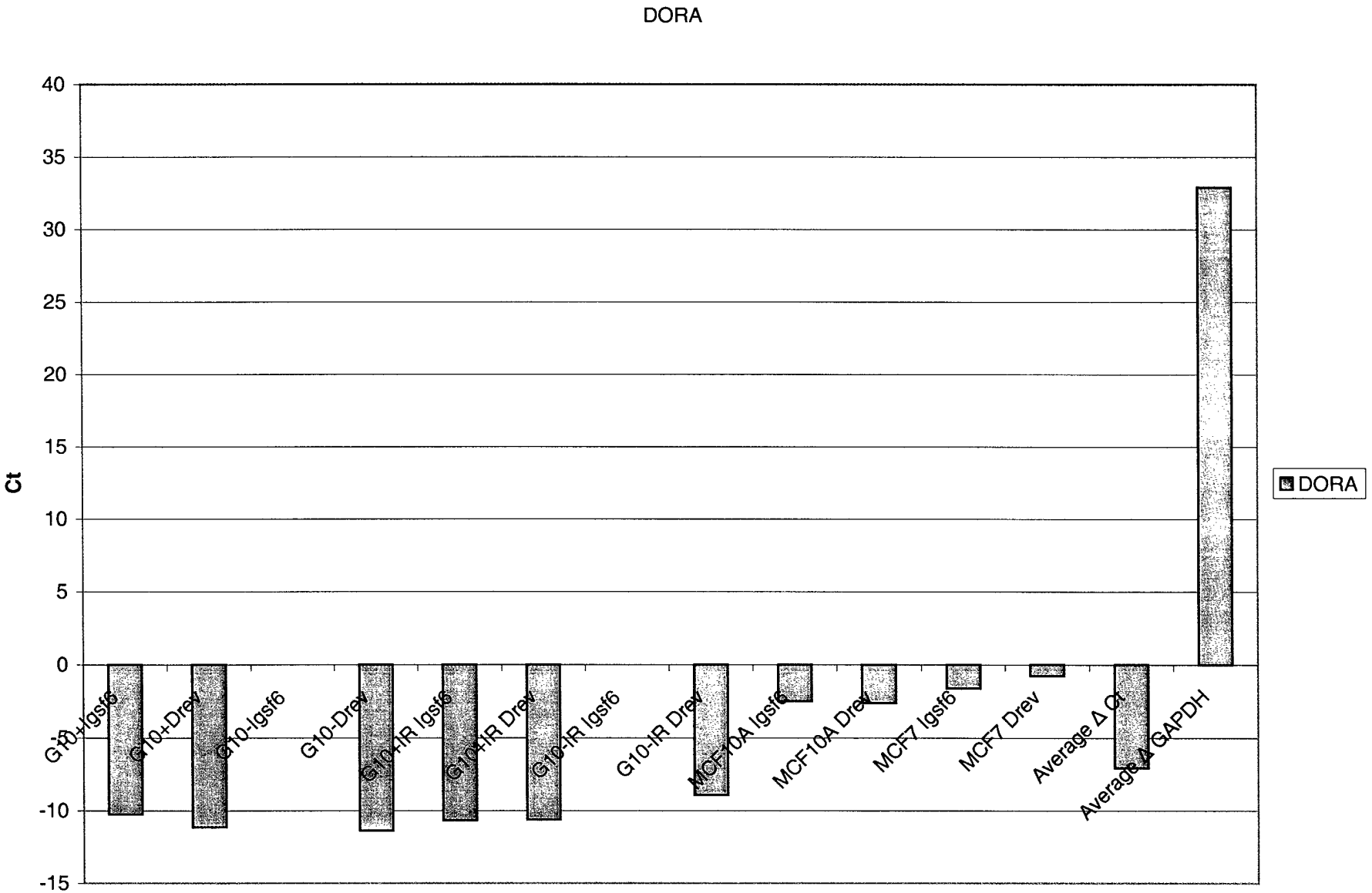
AndrogenR2
CCCATTTCGCTTTTGACACAA
AndrogenR3
GCCCATTTCGCTTTTGACA
DORAF1
GAGGCAGGGTCATCCTTGC
DORAF2
GAGCCAACTAGAGGCAGGGTC
DORAF3
GCCAACTAGAGGCAGGGTCA
DORAR1
CCCACTTGCCACCTACGTTT
DORAR2
TCCCACTTGCCACCTACGTT
DORAR3
CTCCCACTTGCCACCTACGT
CKF1
TGCTACCATGGGCACCAGT
CKF2
TTGCTACCATGGGCACCAGT
CKF3
TTGCTACCATGGGCACCAG
CKR1
GCACACACTTTCTGCCGGT
CKR2
GCACACACTTTCTGCCGGTT
CKR3
GGCACTCGGCCATGCA
EEF1B2F1
CACAATTTGCGCGCTCTCT
EEF1B2F2
CCACAATTTGCGCGCTCT
EEF1B2F3
CCACAATTTGCGCGCTC
EEF1B2R1
ACCCATGGTGTCGGCTGTA
EEF1B2R2
ACCCATGGTGTCGGCTGT
EEF1B2R3
AACCCATGGTGTCGGCTGTA
L27F1
GCCCCTACAGCCATGCTCT
L27F2
ATCGCCCCTACAGCCATG
L27F3
TCAGATCGCCCCTACAGCC
L27R1
CATGGCAGCTGTCACTTTGC
L27R2
CCCATGGCAGCTGTCACTT

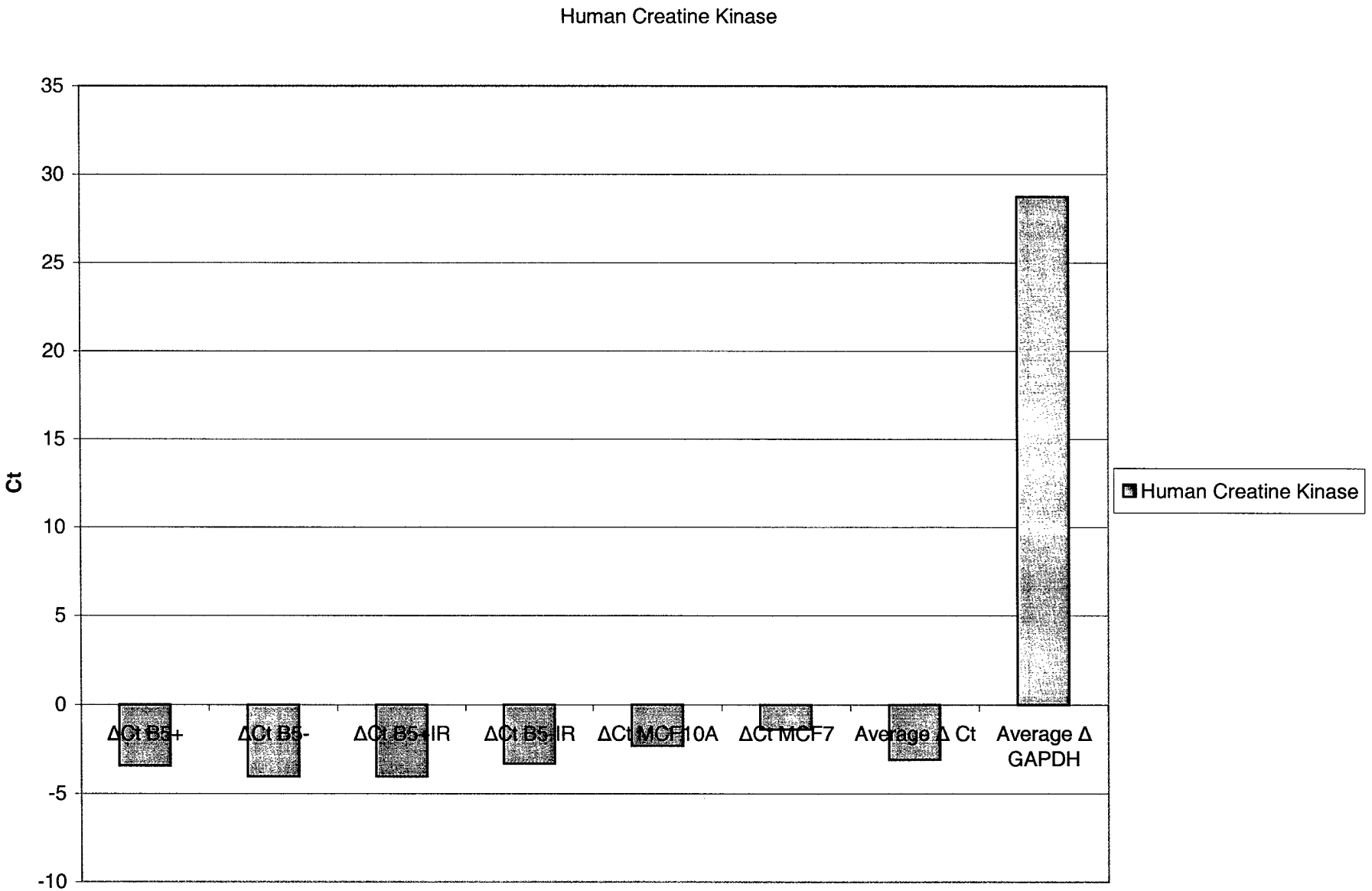
L27R3
TCTTGGCGATCTTCTTCTTGC

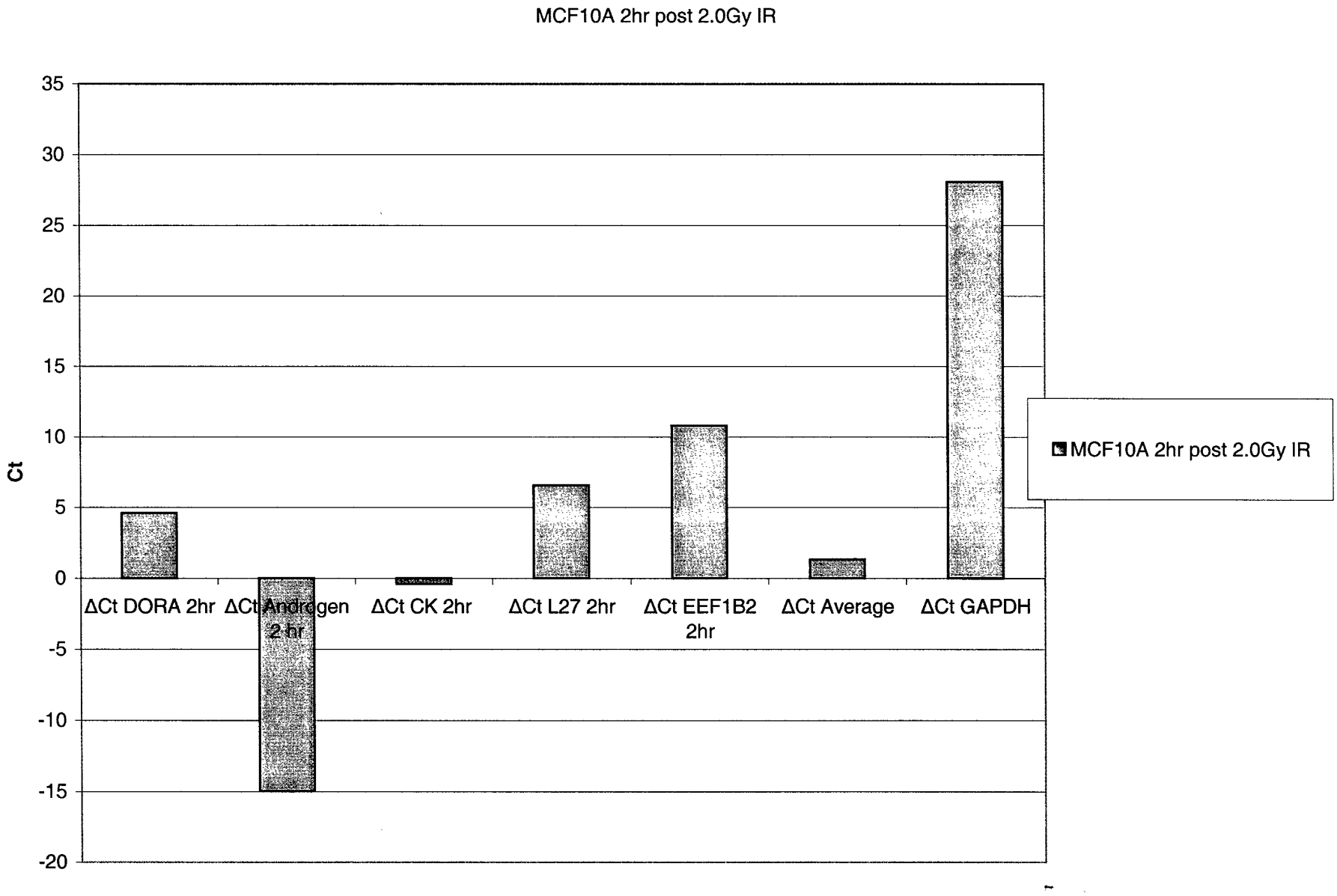
Real-Time PCR Probes (concentration in reaction at 100nM)
Igsf6
6FAM-TGCCCTTCTGAGCAACCAACATGC-TAMRA
Androgen
6FAM-AGCAGAGTGCCCTATCCCAGTCCCA-TAMRA
DORA
6FAM-CTTGTCCTCCCCTTTCATCCCTATGTGG-TAMRA
CK
6FAM-TCCTGACCACCGGGTACCTGCTG-TAMRA
EEF1B2
6FAM-TCTGCTGCTCCCCAGCTCTCGG-TAMRA
L27
6FAM-TGGCTGGAATTGACCGCTACCCC-TAMRA

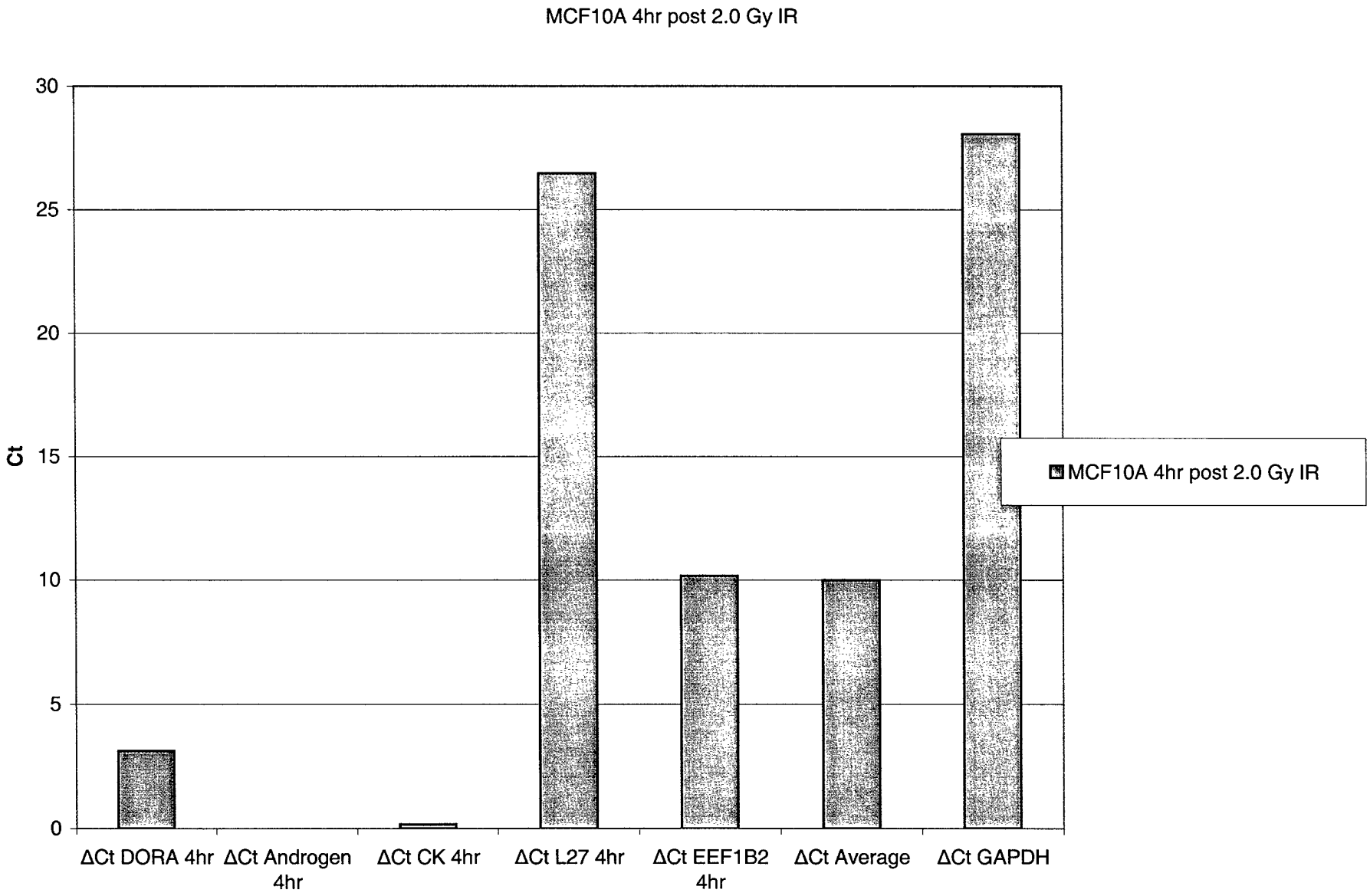

L27
50
45
40
35
30
25
20
15
10
5
0
Ct
A4-
A4+
A4-IR
A4+IR
MCF10A
MCF7
Δ Ct Average
Δ Ct Average GAPDH
L27


EEF1B2
30
25
20
15
10
5
0
Ct
ΔCt E8+
ΔCt E8-
ΔCt E8+IR
ΔCt E8-IR
ΔCt MCF10A
ΔCt MCF7
ΔCt Average
ΔCt GAPDH
EEF1B2

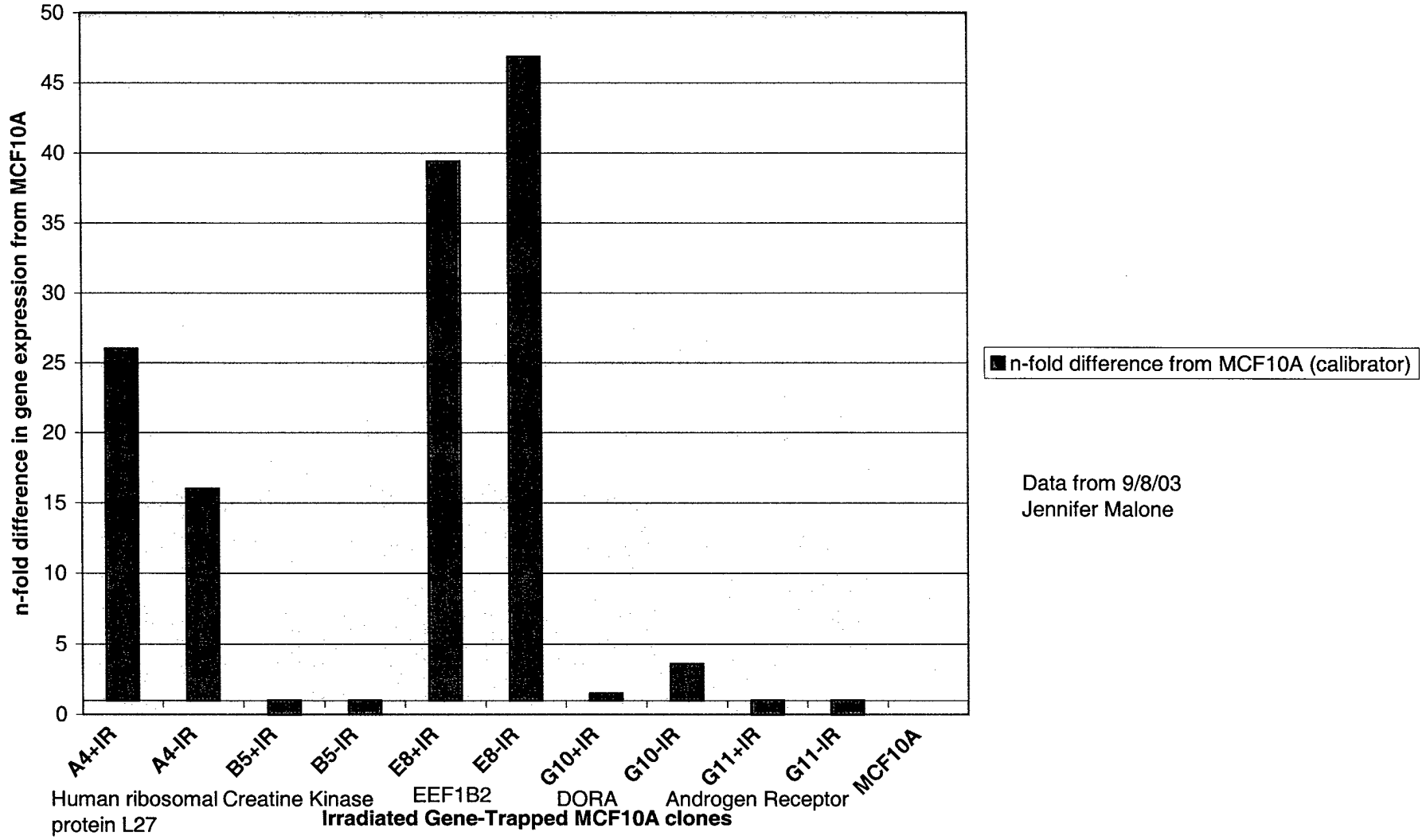

DORA
40
35
30
25
20
15
10
5
0
-5
-10
-15
Ct
G10+Igsf6
G10+Drev
G10-Igsf6
G10-Drev
G10+IR Igsf6
G10+IR Drev
G10-IR Igsf6
G10-IR Drev
MCF10A Igsf6
MCF10A Drev
MCF7 Igsf6
MCF7 Drev
Average Δ Ct
Average Δ GAPDH
DORA


Human Creatine Kinase
35
30
25
20
15
10
5
0
-5
-10
Ct
ΔCt B5+
ΔCt B5-
ΔCt B5+IR
ΔCt B5-IR
ΔCt MCF10A
ΔCt MCF7
Average Δ Ct
Average Δ GAPDH
Human Creatine Kinase

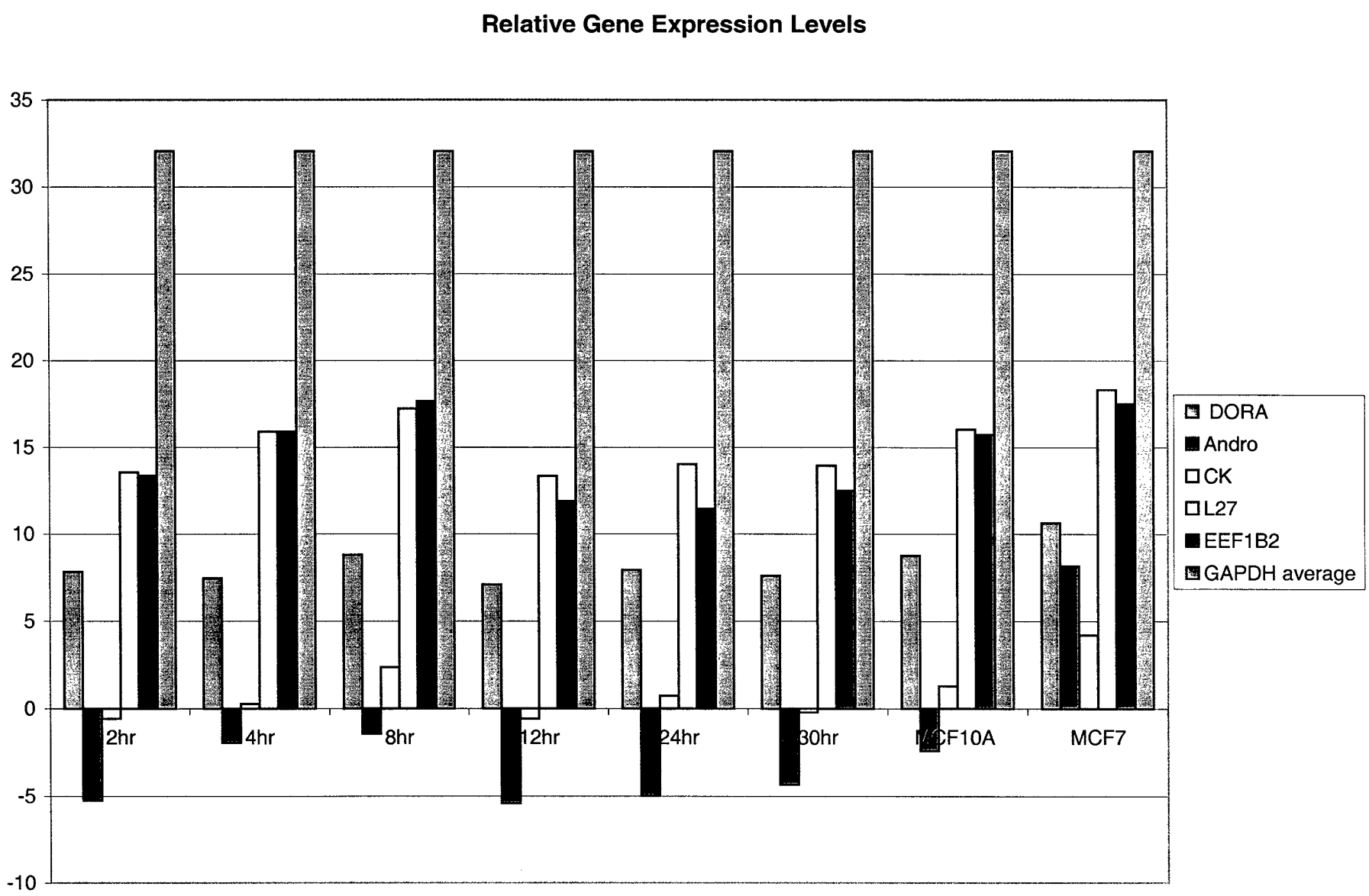

MCF10A 2hr post 2.0Gy IR
35
30
25
20
15
10
5
0
-5
-10
-15
-20
Ct
ΔCt DORA 2hr
ΔCt Androgen 2 hr
ΔCt CK 2hr
ΔCt L27 2hr
ΔCt EEF1B2 2hr
ΔCt Average
ΔCt GAPDH
MCF10A 2hr post 2.0Gy IR

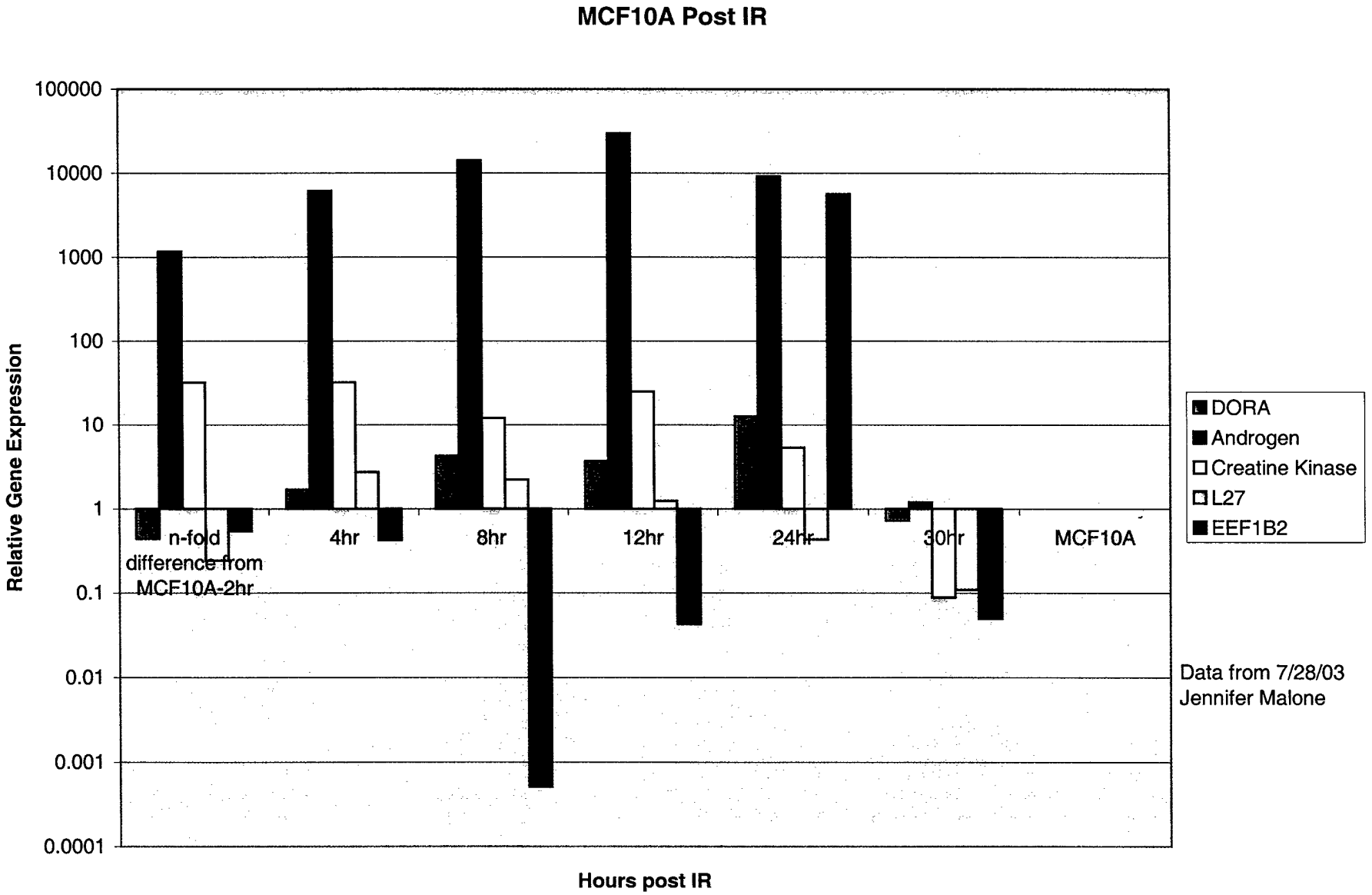

MCF10A 4hr post 2.0 Gy IR
30
25
20
15
10
5
0
Ct
ΔCt DORA 4hr
ΔCt Androgen 4hr
ΔCt CK 4hr
ΔCt L27 4hr
ΔCt EEF1B2 4hr
ΔCt Average
ΔCt GAPDH
MCF10A 4hr post 2.0 Gy IR

n-fold difference from MCF10A (calibrator)

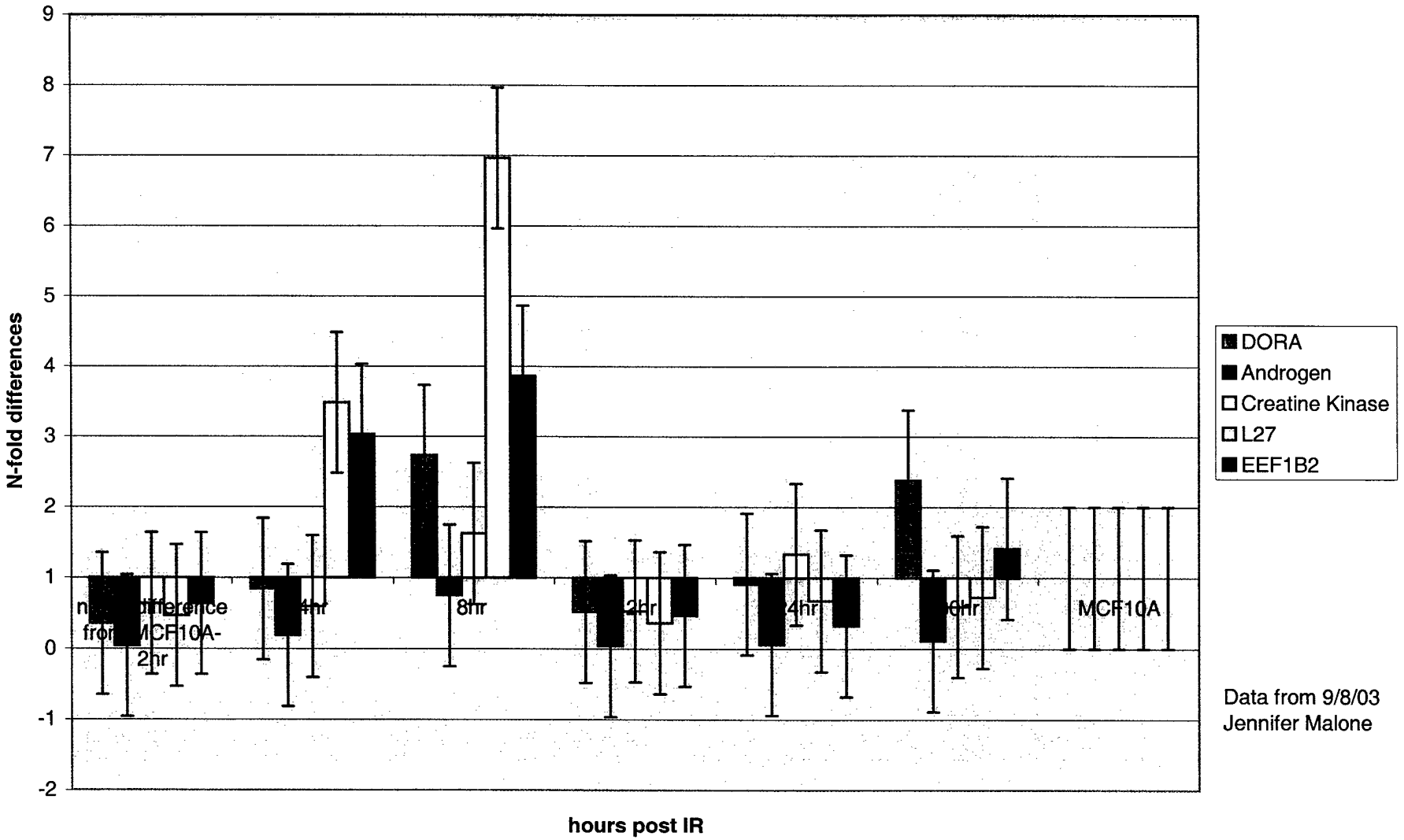


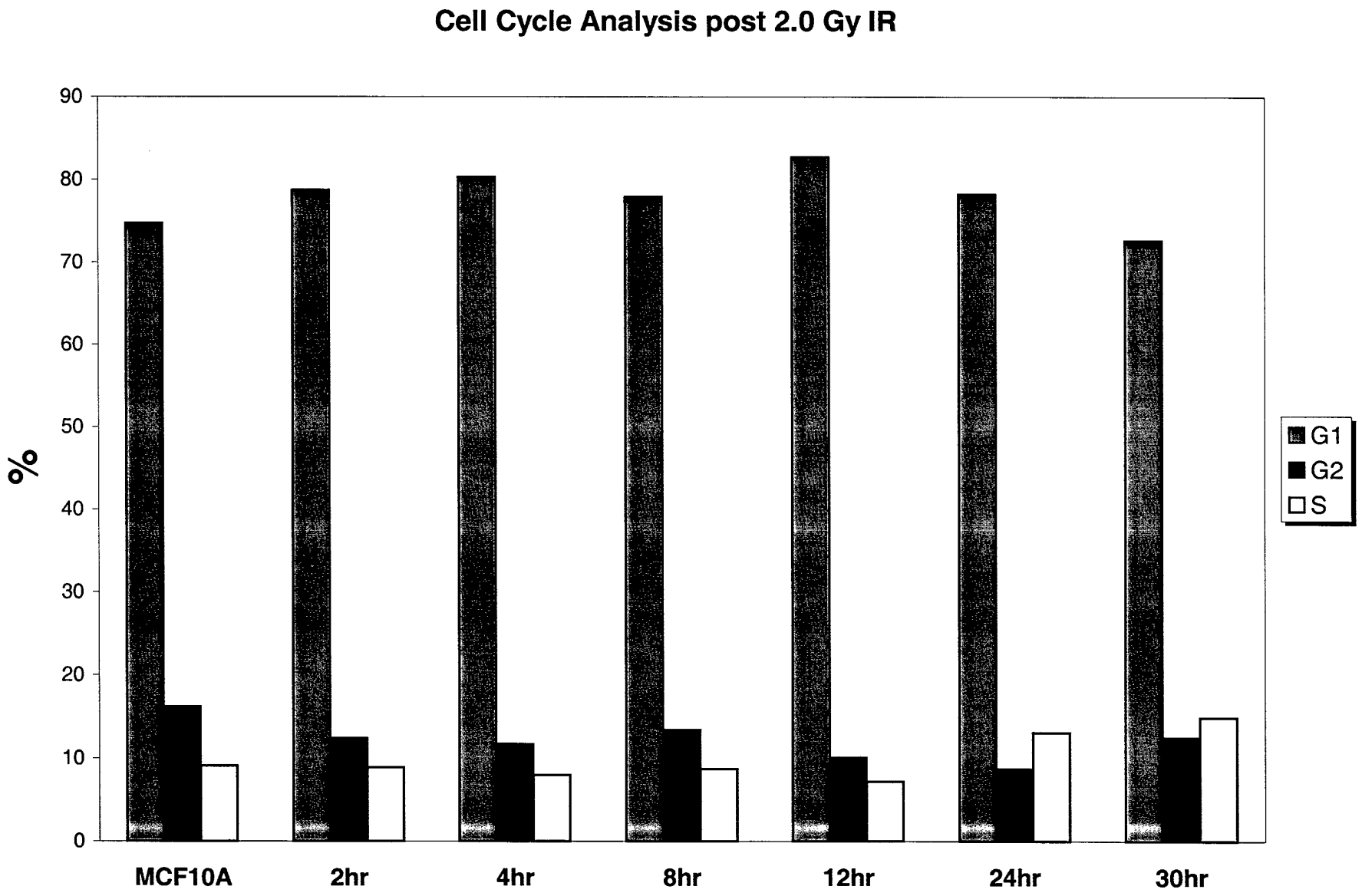
Data from 9/8/03
Jennifer Malone


Relative Gene Expression Levels
35
30
25
20
15
10
5
0
-5
-10
2hr
4hr
8hr
12hr
24hr
30hr
MCF10A
MCF7
DORA
Andro
CK
L27
EEF1B2
GAPDH average

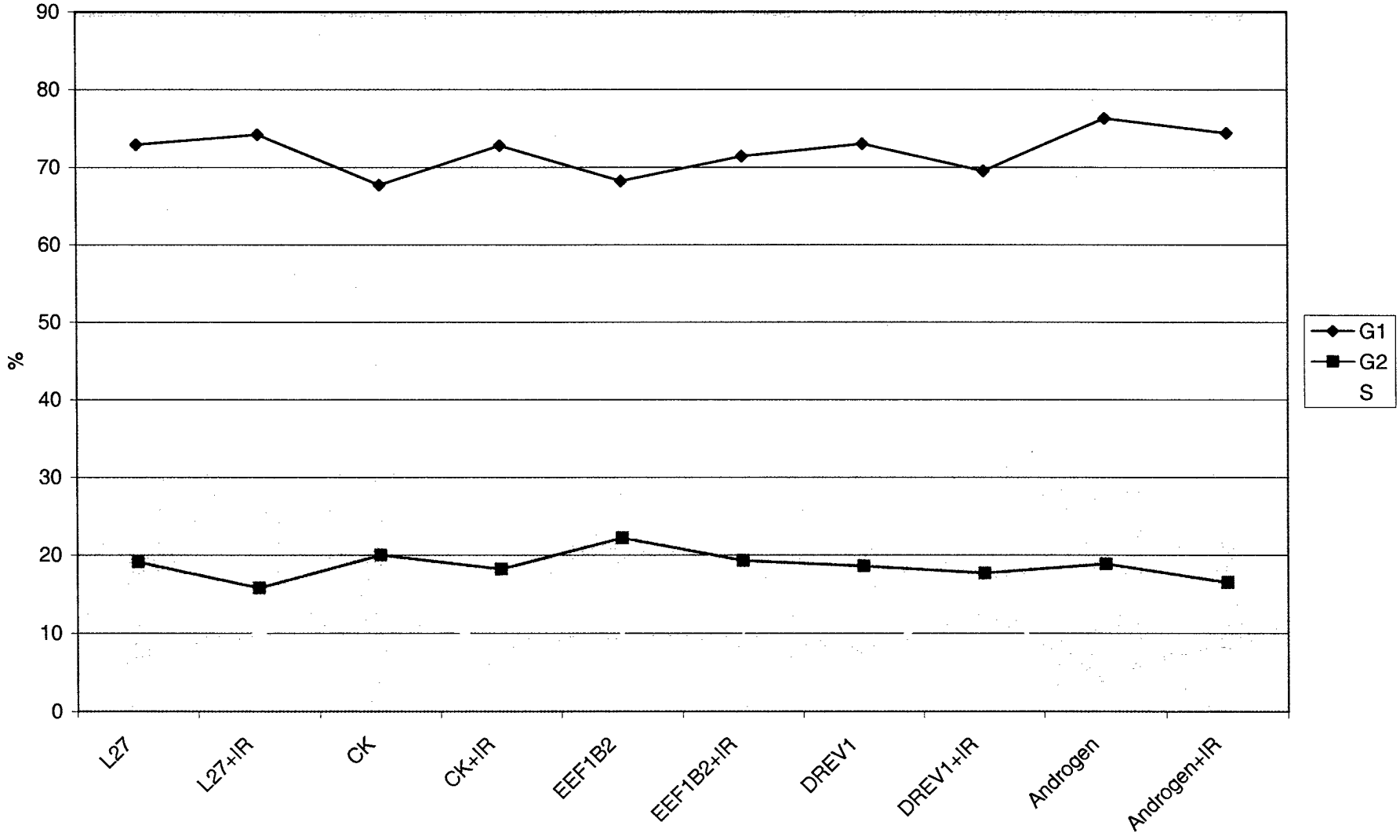

MCF10A Post IR
100000
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
Relative Gene Expression
n-fold difference from MCF10A-2hr
4hr
8hr
12hr
24hr
30hr
MCF10A
Hours post IR
DORA
Androgen
Creatine Kinase
L27
EEF1B2
Data from 7/28/03
Jennifer Malone

Gene Expression post IR




Cell Cycle Analysis post 2.0 Gy IR
%
90
80
70
60
50
40
30
20
10
0
MCF10A
2hr
4hr
8hr
12hr
24hr
30hr
G1
G2
S

Cell Cycle Analysis of Potential Radiation Response Genes



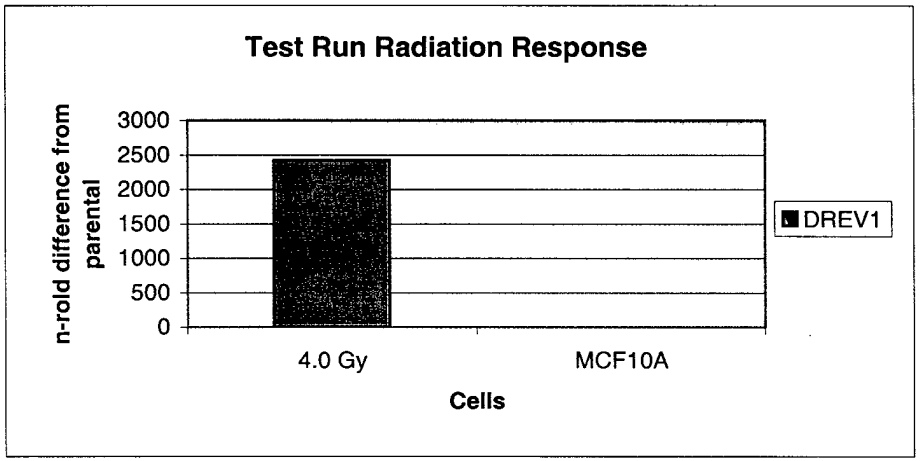
	4.0Gy	MCF10A
DREV1	27.2269	35.3012
DREV1	25.1861	37.5656
DREV1	23.1694	
average	25.19413	36.4334

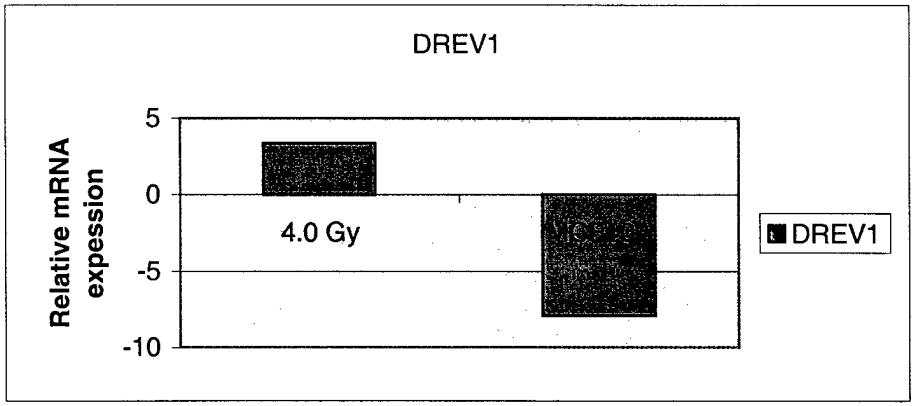
	GAPDH			average
80 pg	34.9355	34.223	36.1337	35.0974
400 pg	31.7649	30.4173	30.8294	31.00387
2 ng	28.0202	27.8527	27.8083	27.89373
10 ng	24.781	24.1228	24.4636	24.4558
50 ng	23.3839	23.8954	25.3165	24.1986

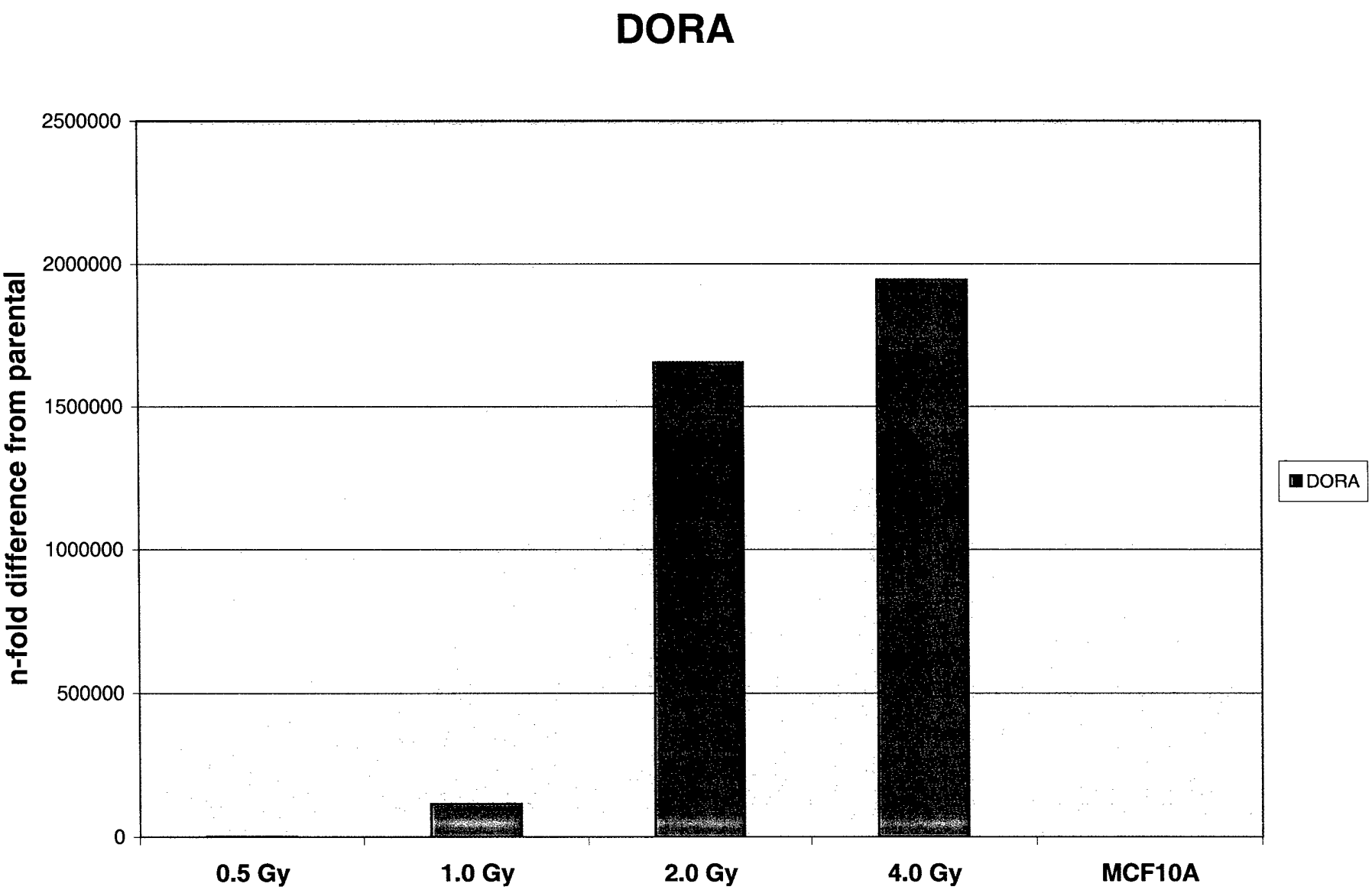
deltadeltaCt	
4.0 Gy	3.335747
MCF10A	-7.90352

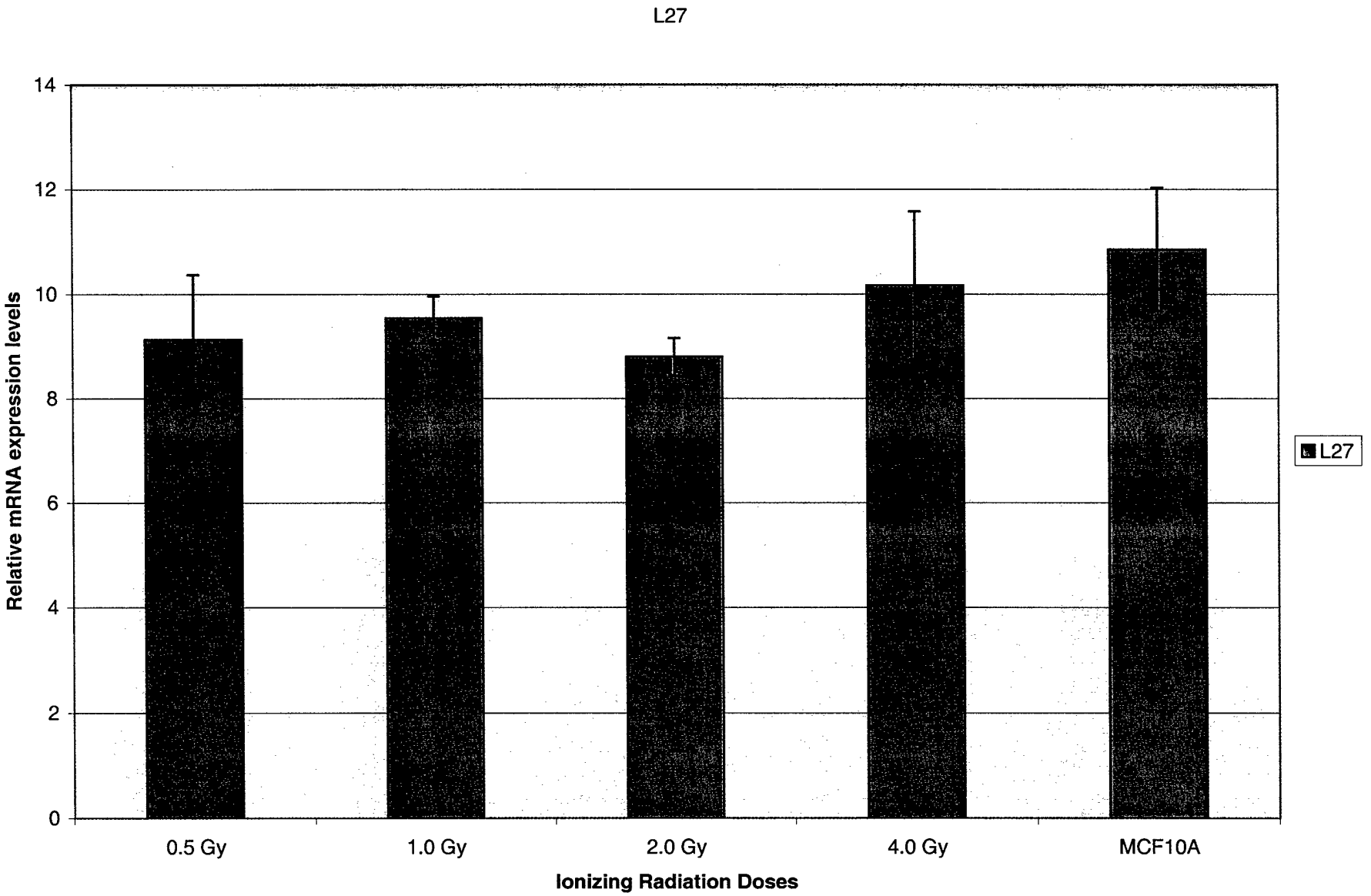
delta Ct GAPDH
28.52988

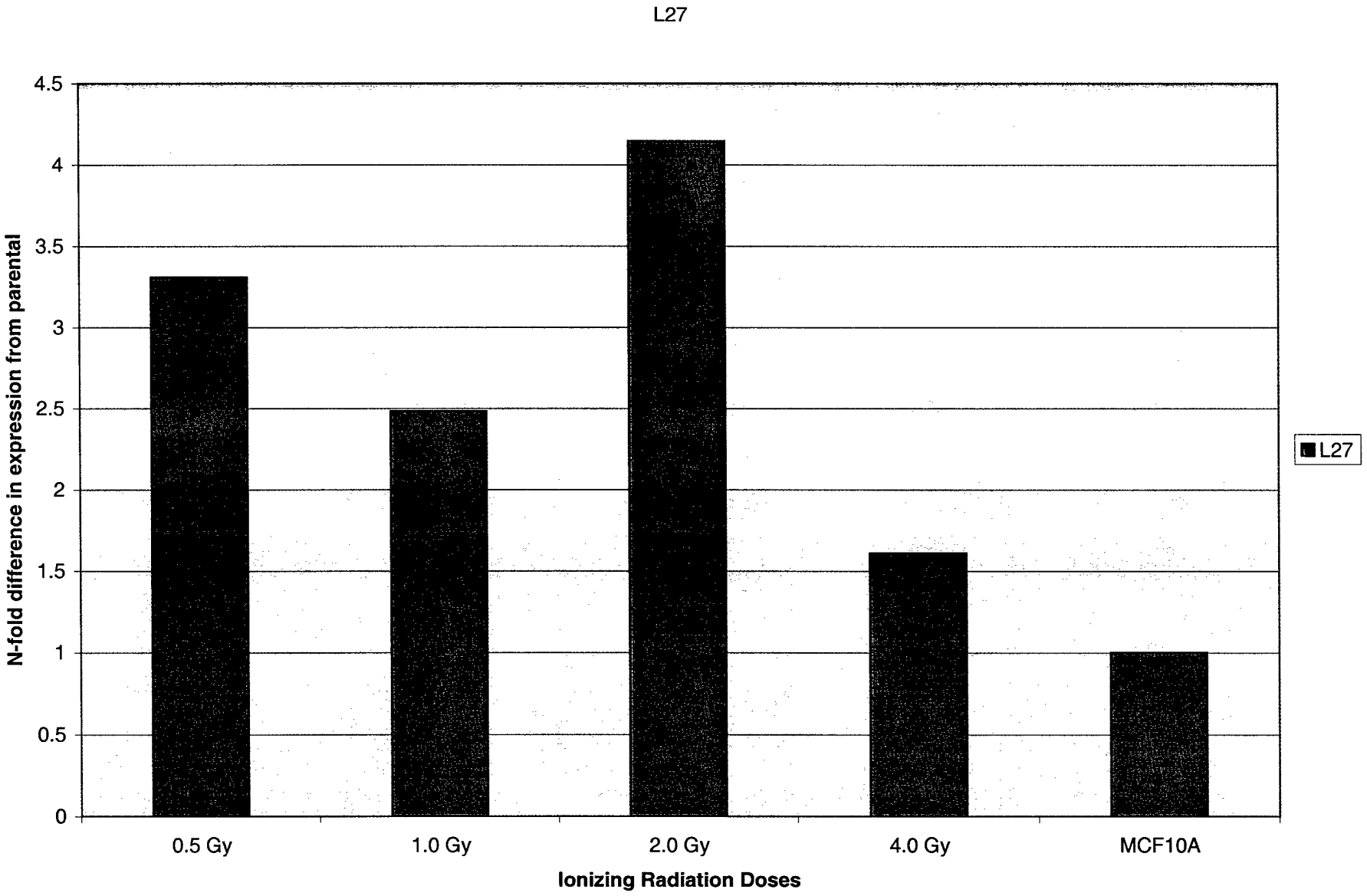
n-fold differences	
4.0 Gy	2417.444
MCF10A	1

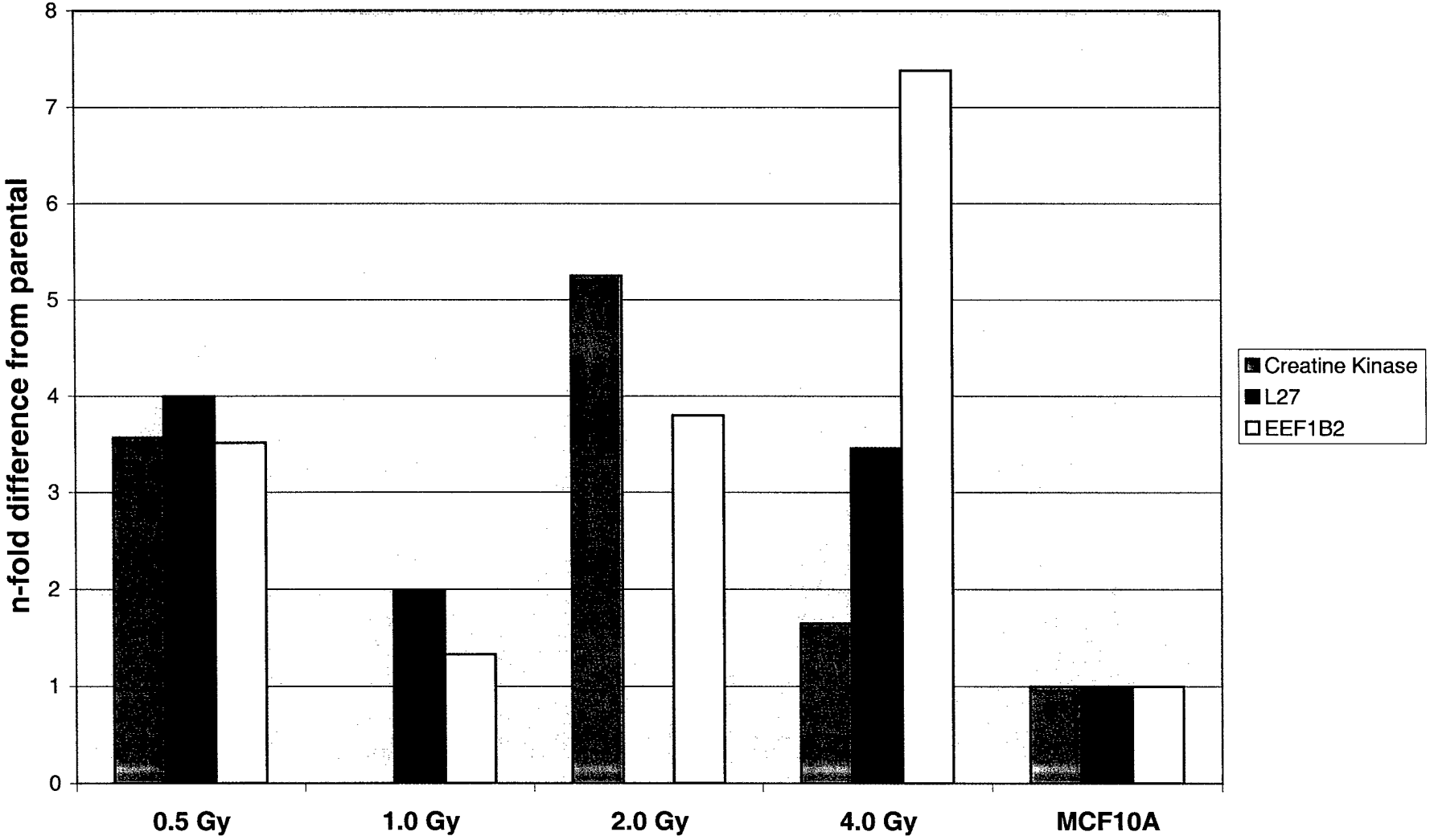


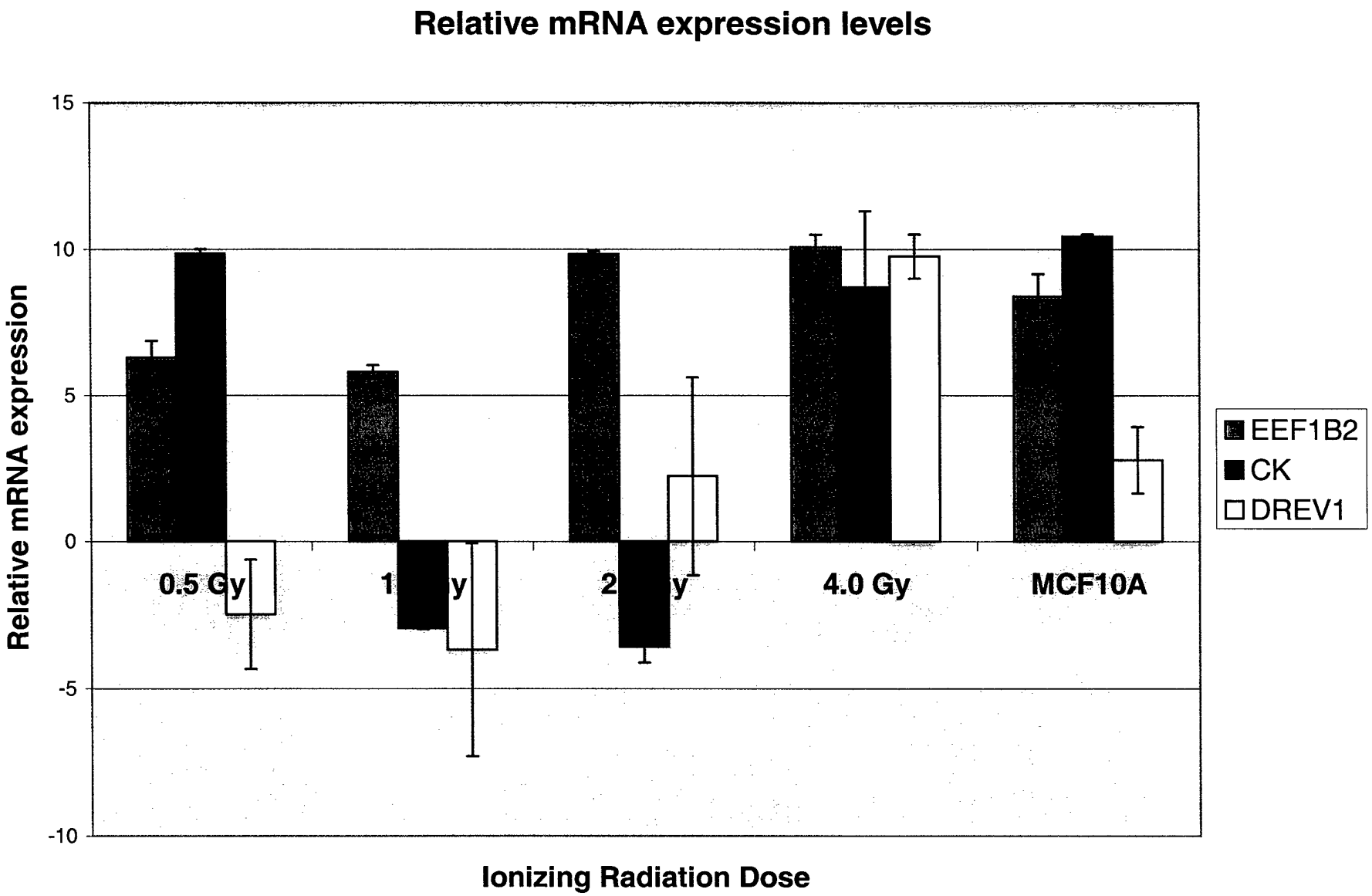


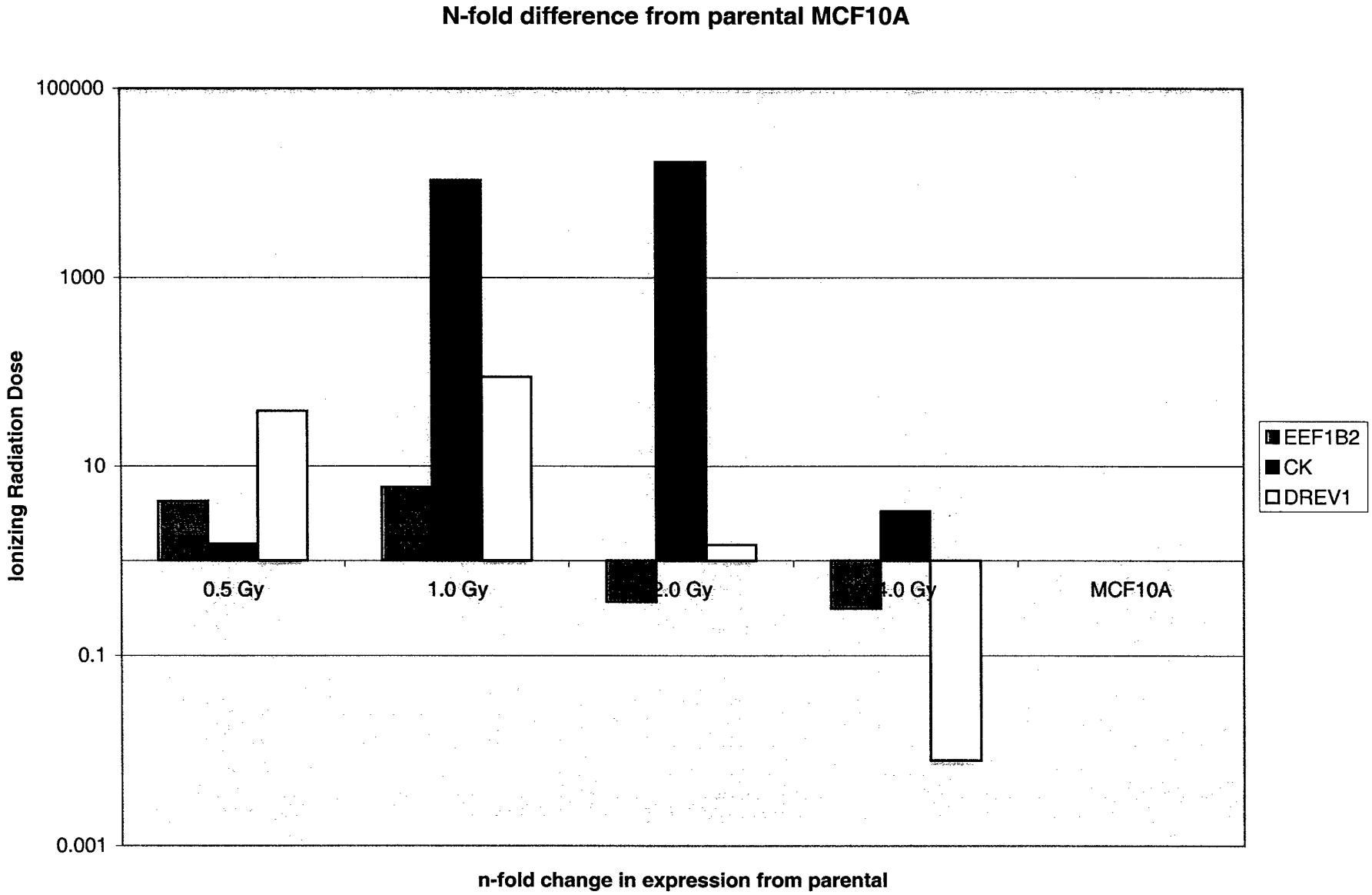

DORA
2500000
2000000
1500000
1000000
500000
0
n-fold difference from parental
0.5 Gy
1.0 Gy
2.0 Gy
4.0 Gy
MCF10A
DORA

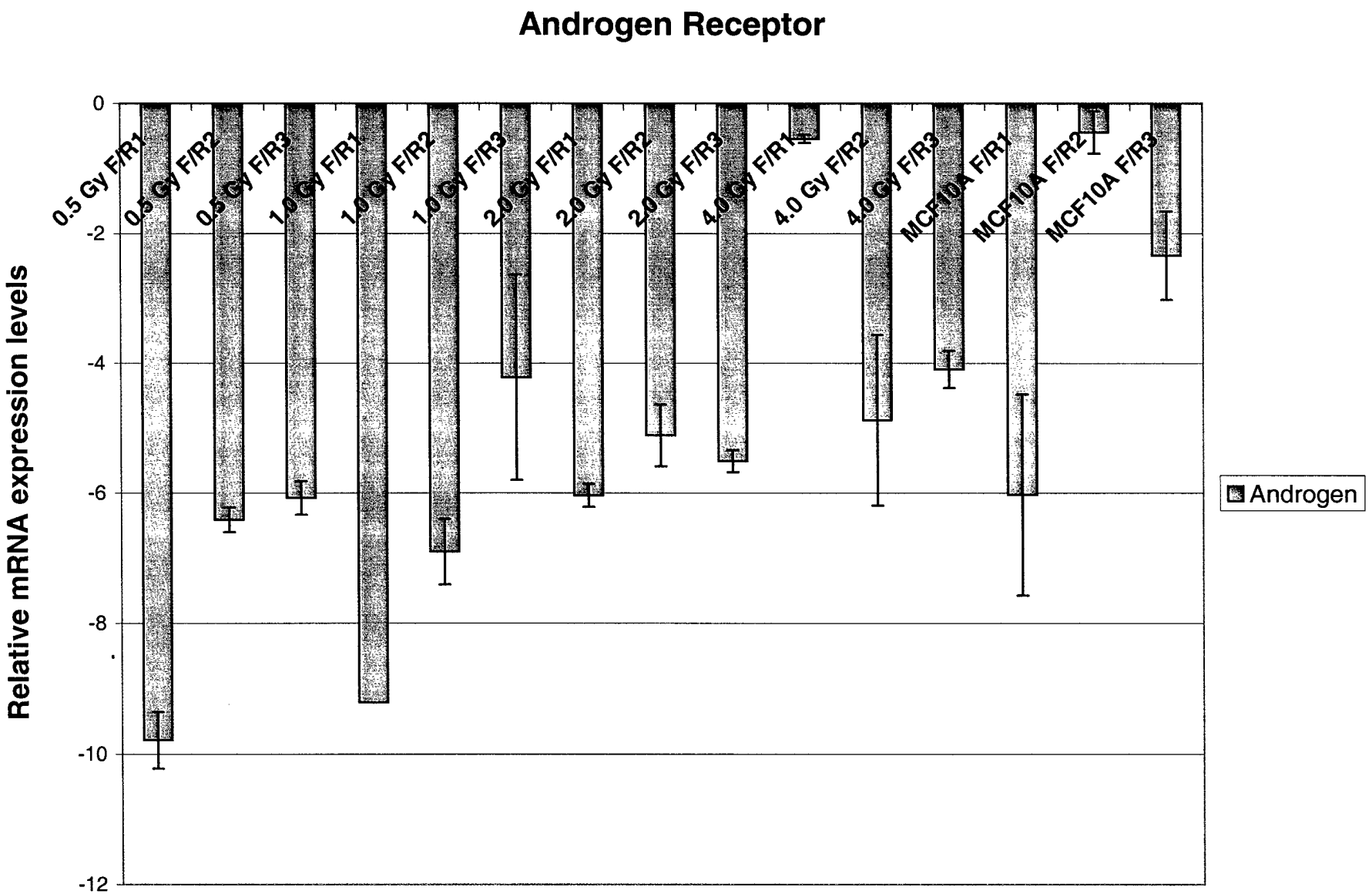

L27
14
12
10
8
6
4
2
0
Relative mRNA expression levels
0.5 Gy
1.0 Gy
2.0 Gy
4.0 Gy
MCF10A
Ionizing Radiation Doses
L27

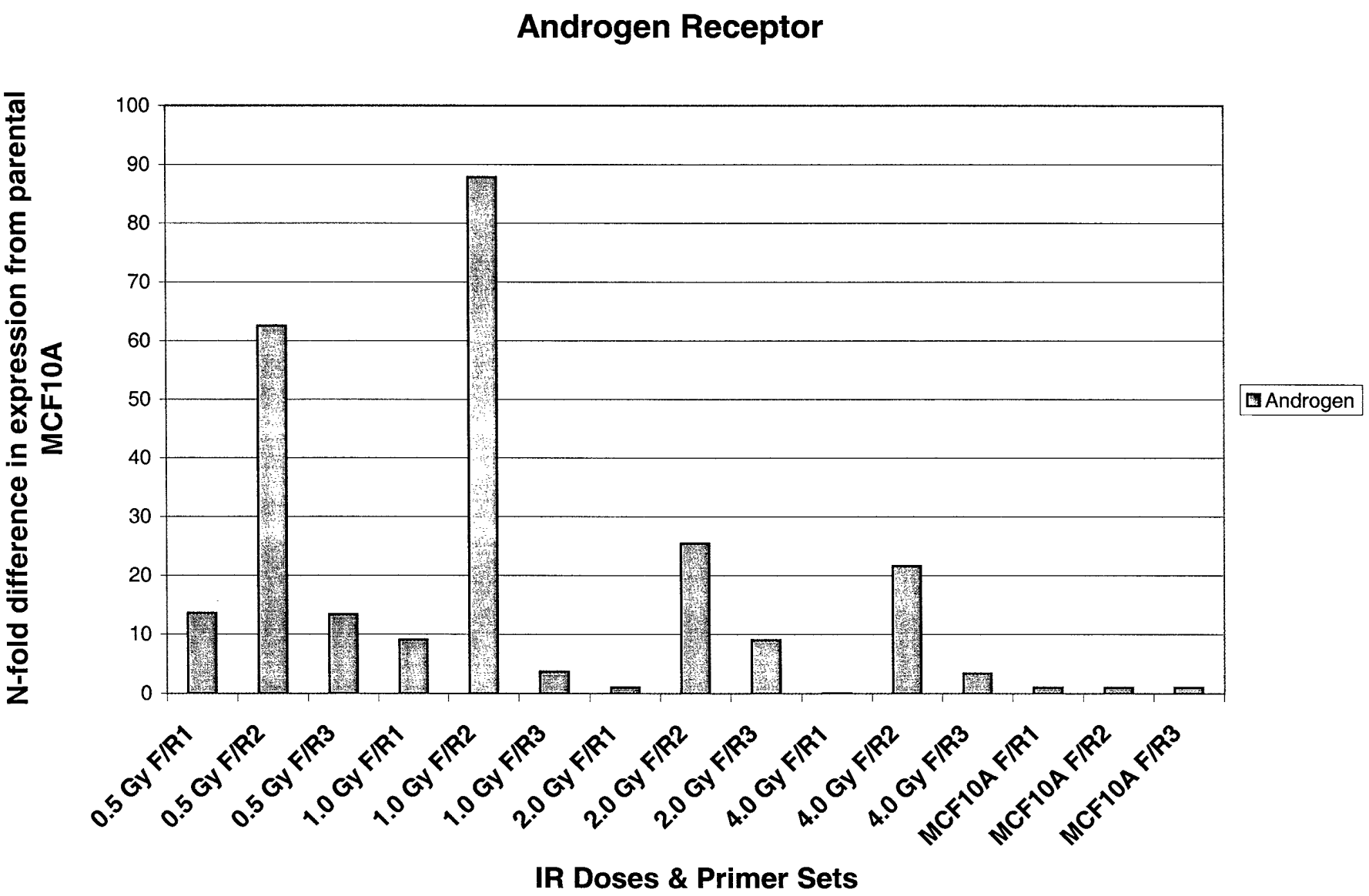

L27
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
N-fold difference in expression from parental
0.5 Gy
1.0 Gy
2.0 Gy
4.0 Gy
MCF10A
Ionizing Radiation Doses
L27

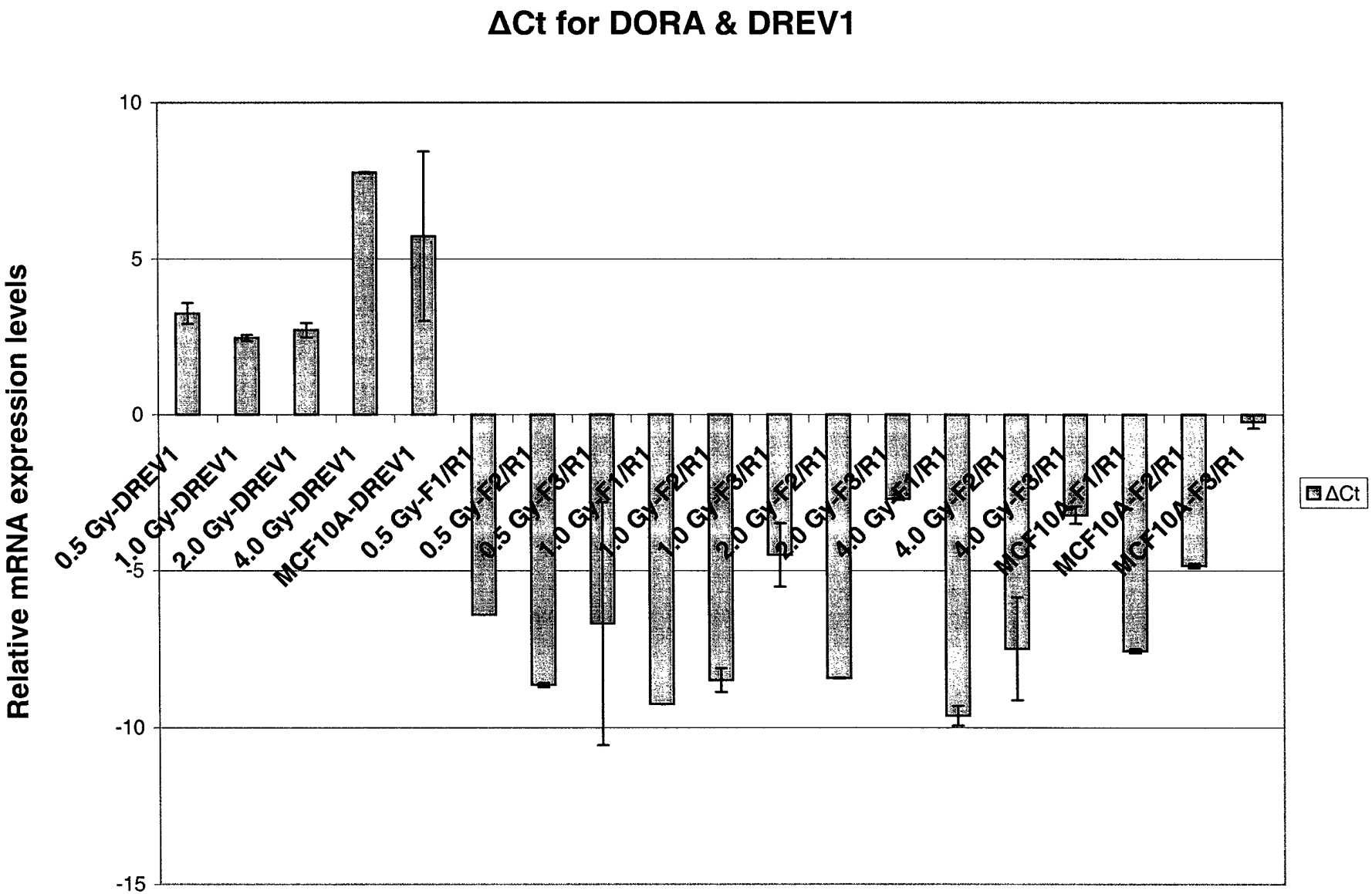

N-Fold Differences from MCF10A
n-fold difference from parental
8
7
6
5
4
3
2
1
0
0.5 Gy
1.0 Gy
2.0 Gy
4.0 Gy
MCF10A
Creatine Kinase
L27
EEF1B2

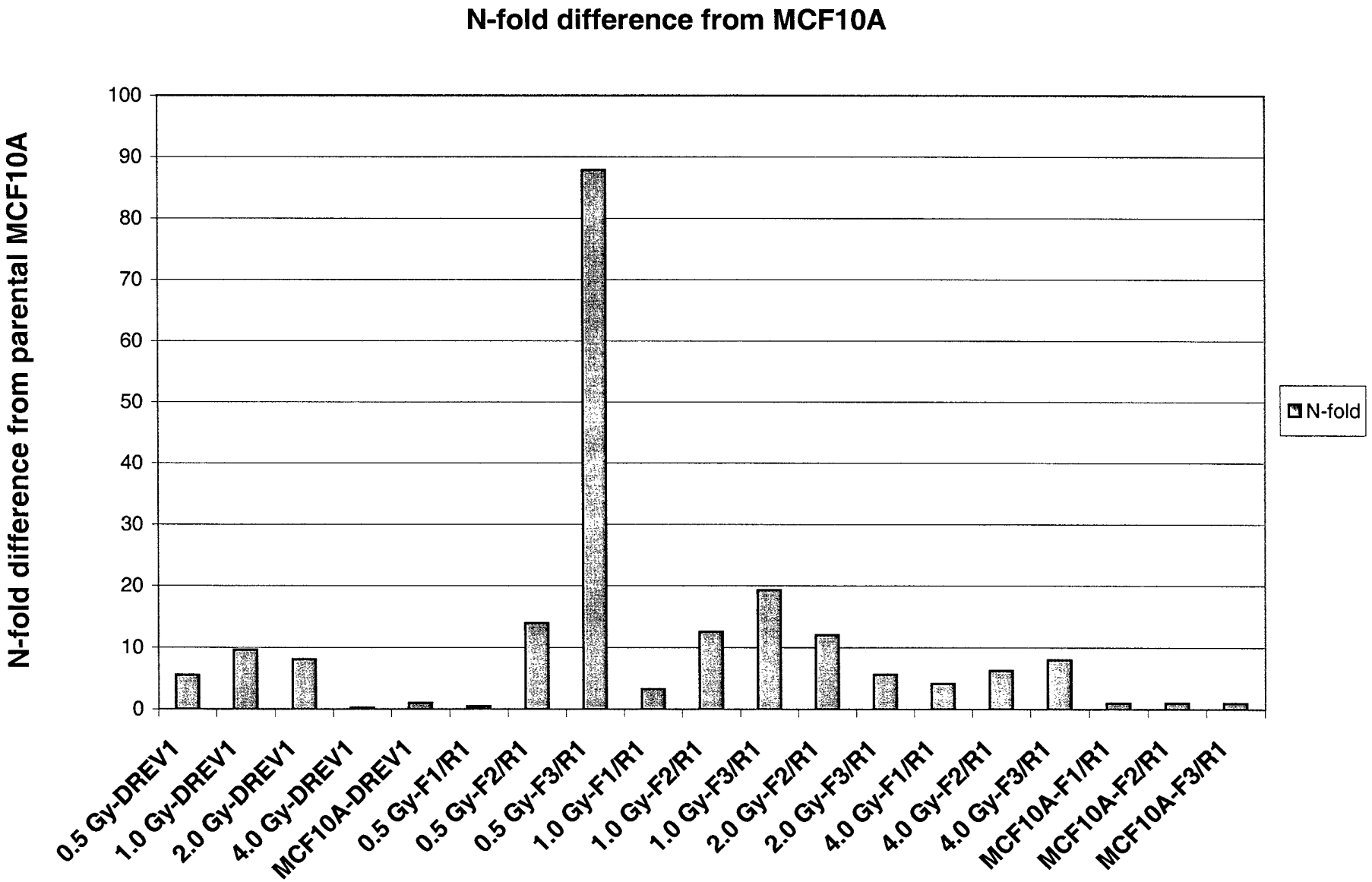

Relative mRNA expression levels
15
10
5
0
-5
-10
Relative mRNA expression
0.5 Gy
1 Gy
2 Gy
4.0 Gy
MCF10A
Ionizing Radiation Dose
EEF1B2
CK
DREV1


N-fold difference from parental MCF10A
100000
1000
10
0.1
0.001
Ionizing Radiation Dose
0.5 Gy
1.0 Gy
2.0 Gy
4.0 Gy
MCF10A
EEF1B2
CK
DREV1
n-fold change in expression from parental


Androgen Receptor
Relative mRNA expression levels
0
-2
-4
-6
-8
-10
-12
0.5 Gy F/R1
0.5 Gy F/R2
0.5 Gy F/R3
1.0 Gy F/R1
1.0 Gy F/R2
1.0 Gy F/R3
2.0 Gy F/R1
2.0 Gy F/R2
2.0 Gy F/R3
4.0 Gy F/R1
4.0 Gy F/R2
4.0 Gy F/R3
MCF10A F/R1
MCF10A F/R2
MCF10A F/R3
Androgen


Androgen Receptor
N-fold difference in expression from parental MCF10A
100
90
80
70
60
50
40
30
20
10
0
0.5 Gy F/R1
0.5 Gy F/R2
0.5 Gy F/R3
1.0 Gy F/R1
1.0 Gy F/R2
1.0 Gy F/R3
2.0 Gy F/R1
2.0 Gy F/R2
2.0 Gy F/R3
4.0 Gy F/R1
4.0 Gy F/R2
4.0 Gy F/R3
MCF10A F/R1
MCF10A F/R2
MCF10A F/R3
IR Doses & Primer Sets
Androgen


ΔCt for DORA & DREV1
Relative mRNA expression levels
10
5
0
-5
-10
-15
0.5 Gy-DREV1
1.0 Gy-DREV1
2.0 Gy-DREV1
4.0 Gy-DREV1
MCF10A-DREV1
0.5 Gy-F1/R1
0.5 Gy-F2/R1
0.5 Gy-F3/R1
1.0 Gy-F1/R1
1.0 Gy-F2/R1
1.0 Gy-F3/R1
2.0 Gy-F2/R1
2.0 Gy-F3/R1
4.0 Gy-F1/R1
4.0 Gy-F2/R1
4.0 Gy-F3/R1
MCF10A-F1/R1
MCF10A-F2/R1
MCF10A-F3/R1
ΔCt


N-fold difference from MCF10A
N-fold difference from parental MCF10A
100
90
80
70
60
50
40
30
20
10
0
N-fold
0.5 Gy-DREV1
1.0 Gy-DREV1
2.0 Gy-DREV1
4.0 Gy-DREV1
MCF10A-DREV1
0.5 Gy-F1/R1
0.5 Gy-F2/R1
0.5 Gy-F3/R1
1.0 Gy-F1/R1
1.0 Gy-F2/R1
1.0 Gy-F3/R1
2.0 Gy-F2/R1
2.0 Gy-F3/R1
4.0 Gy-F1/R1
4.0 Gy-F2/R1
4.0 Gy-F3/R1
MCF10A-F1/R1
MCF10A-F2/R1
MCF10A-F3/R1

Analysis of Novel Radiation Response Genes

Jennifer Malone
Colorado State University
R770 Student Seminar
March 18, 2004

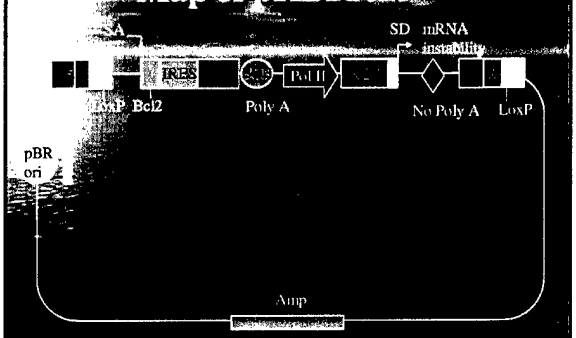
Outline

- Intro
- Overview of novel radiation response gene functions
- Current Research Findings
- Future Experiments

Intro

- Gene-Trapped MCF10A cell line used to identify 5 novel radiation response genes.
- Gene Trapping is a form of insertional mutagenesis that allows the gene where random retroviral integration occurred to be monitored via a reporter, GFP.
- The endogenous trapped gene that is found to respond to radiation can then be sequenced & further analyzed.

Map of pRET1023



Current Research Findings

- Genes found to respond to radiation:

Human DORA reverse strand protein 1 (DREV1)

Human Androgen Receptor

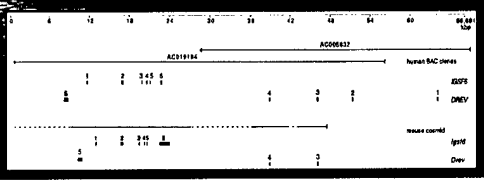
Human Eukaryotic Translation Elongation Factor 1 Beta 2

Human Creatine Kinase Gene

Human Ribosomal Protein L27

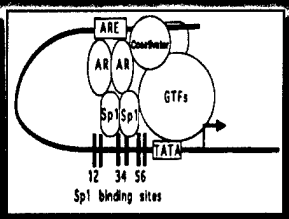
Human DORA reverse strand protein 1 (DREV1)

- Novel member of immunoglobulin superfamily
- Associated w/ inflammatory bowel disease locus on chromosome 16
- Type I membrane protein w/ single Ig V_1J-type loop
- The small gene DORA is embedded in intron 4 on the complement strand
- Has homology to DNA methyltransferases



Androgen Receptor (AR)

- p21 (WAF1, CIP1, SDI1, CAP20) gene
- AR and transcription factor Sp1 interact
- Prostate-specific antigen (PSA)
- Kallikrein gene family
- Regulates several cell cycle-regulatory molecules such as CDK2 & 4 that promote cell proliferation
- Inhibits p16 family of CDK inhibitors

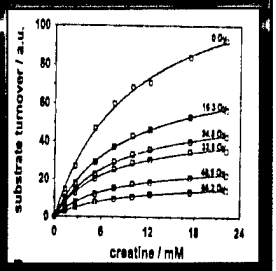


Human Eukaryotic Translation Elongation Factor 1 Beta 2

- Located on chromosome 2
- Expressed in a wide range of tissue types, expected of a single gene encoding protein predicted to be essential
- rare, recessive, juvenile-onset motor neuron disease/amyotrophic lateral sclerosis (ALS2) mapped to this region
- Elongation factors may constitute up to 5% of the total cellular protein in actively proliferating cells--tumor & cultured cells express levels up to 20-fold higher than normal

Human Creatine Kinase (CK)

- Inactivated by free radical damage – indirect radiation effects
- Inactivation of CK by ROS might be involved in energy metabolic regulation in cancer
- CK activity lower in cancer tissues than in normal tissues
- E6 protein from papilloma virus promotes degradation of p53 resulting in increased CK.



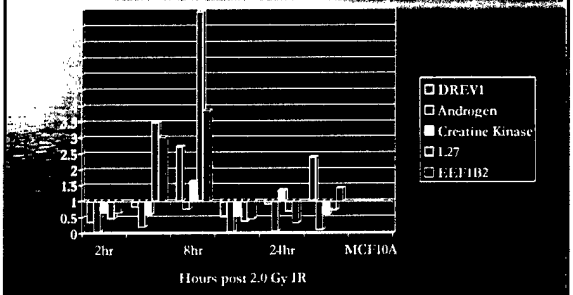
Human Ribosomal Protein L27

- Ribosome responsible for protein synthesis
- Located on chromosome 17

- The gene-trapped clones were expanded and harvested for RNA extraction.
- RT-PCR was performed and the cDNA was used in a real-time PCR reaction.

Relative Gene Expression of 2.0 Gy Irradiated Gene-Trapped MCF10A Clones

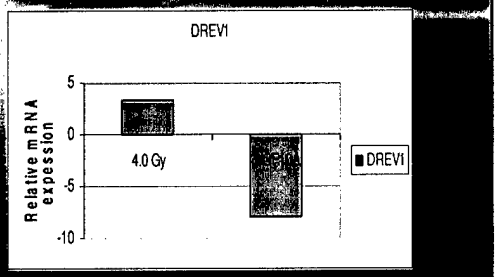
Relative Gene Expression of MCF10A cells collected post 2.0 Gy IR



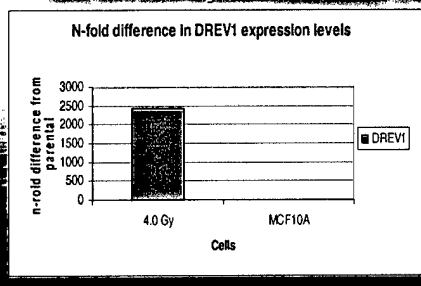
Experiments in Progress

- Analyze the 5 genes identified through the use of gene trapping in the parental MCF10A cell line to verify that a radiation response is indeed being seen.
- Analyze the MCF10A cells at the following doses of IR: 0.5 Gy, 1.0 Gy, 2.0 Gy, and 4.0 Gy.
- At each dose level the MCF10A cells will be analyzed by real-time PCR for the mRNA expression levels of the 5 genes.

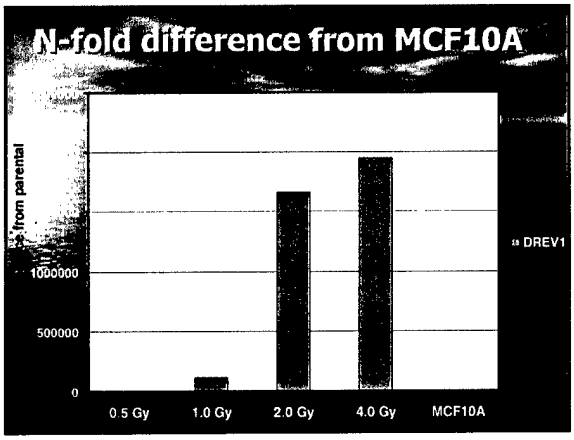
Relative DREV1 mRNA expression after 4.0 Gy IR



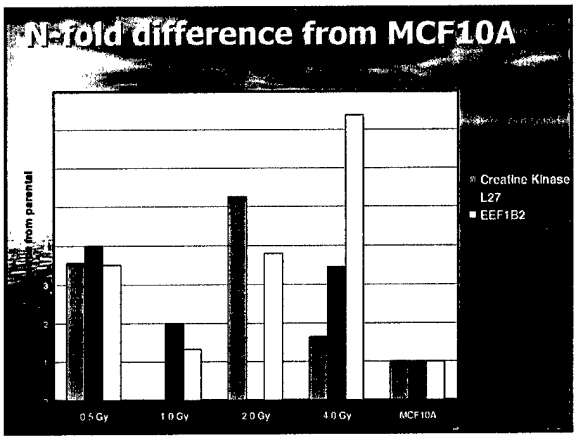
N-fold difference in DREV1 expression levels compared to MCF10A after 4.0 Gy IR

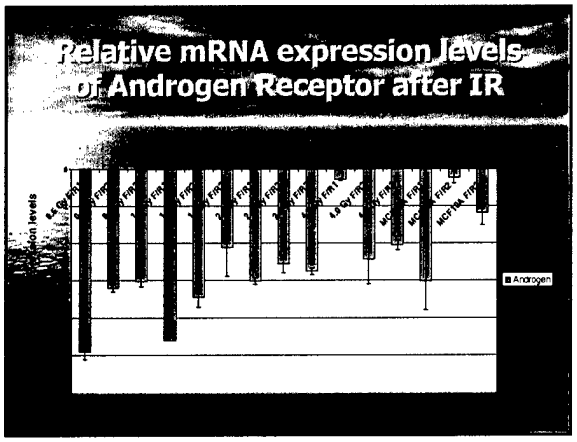


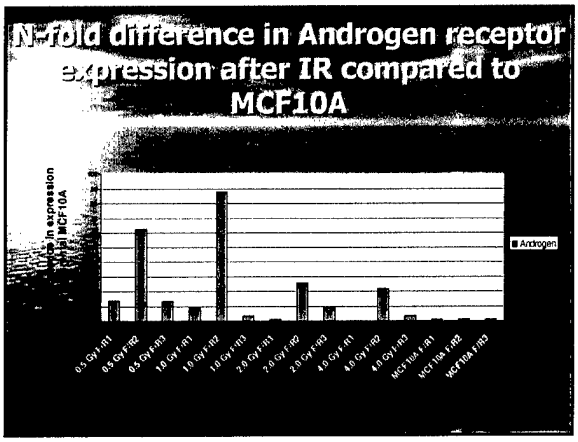
N-fold difference from MCF10A

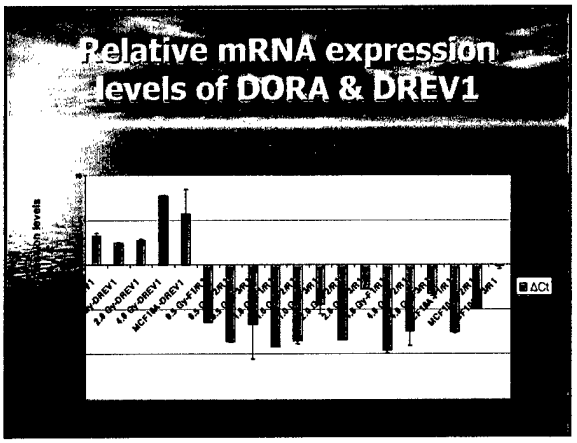


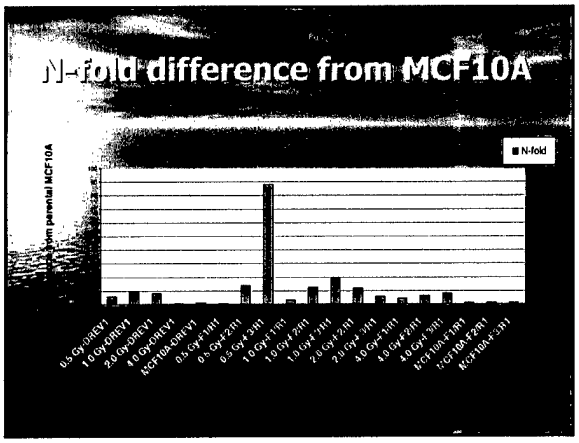
N-fold difference from MCF10A









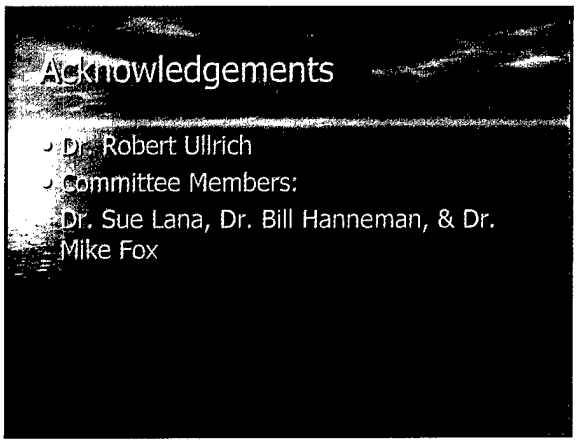


Future Experiments

- For each radiation response gene: at the IR dose with the highest response a time course of gene expression will be done at various time points following irradiation.
- The time points will be at 2, 4, 8, 12, 24, and 30 hours post IR.

On the agenda next . . . . .

- Radiation response time course real-time PCR experiment


Acknowledgements
Dr. Robert Ullrich
Committee Members:
Dr. Sue Lana, Dr. Bill Hanneman, & Dr. Mike Fox

Novel radiation response genes identified in MCF10A gene-trapped cells.

Jennifer Malone and Robert Ullrich
Department of Environmental and Radiological Health Sciences, Colorado State University
Graduate Student, 491-7497, Jennifer.Malone@ColoState.EDU

Objective/Hypothesis: In this study, we have established an assay to identify novel genes that are affected by gamma irradiation and to characterize their function and role in early breast carcinogenesis. We hypothesize that the mutation of these genes or their abnormal expression in response to gamma irradiation is one of the causes of breast carcinogenesis.
Specific Aims: The specific aims of this study are:
1. To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones; 2. To determine the effect of gamma irradiation on the expression of the reporter, green fluorescent protein (GFP); 3. To characterize the effect of gamma irradiation on the transformation of human mammary epithelial cells; 4. To identify the trapped genes affected by gamma irradiation in breast epithelial cells.
Methods: We plan to establish an assay that will allow us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. This will allow us to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Gene-trapped clones that are affected by radiation will be isolated and further analyzed by real-time PCR and compared to the parental to verify that a radiation response is being seen.
Results: The MCF10A gene-trapped library has been established and basal GFP levels have been measured. Gamma irradiation of the single cell gene-trapped clones at both 1.0 and 2.0 Gy has been performed. Clones that were either up- or down-regulated at least 2-fold in response to the radiation treatment have been expanded and analyzed by 3' RACE and sequencing. The five radiation response genes identified have been analyzed by real time PCR and cell cycle analysis.
Study Design: Using the poly-A trap retrovirus RET, we have established a gene-trapped library of clones from human mammary epithelial cells (MCF10A). This provides a strong base for the identification of novel genes that may be involved in essential signaling pathways in human mammary epithelial cells. The reporter gene GFP, which has been incorporated into the genome of the cells, monitors the expression level of the endogenous trapped genes. We will compare basal GFP expression before and after exposure to varying low doses of gamma radiation (0-4 Gy) using replica plates of MCF10A gene-trapped clones. We will then identify the genes involved by using 3' RACE and sequencing. The identified radiation response gene's mRNA levels will be analyzed by real-time PCR analysis and compared to the parental MCF10A cell line after varying doses and time points following ionizing radiation.
Conclusions: This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation-responsive genes that can lead to breast cancer as well as identify new markers for early detection of breast cancer. This study will focus on the identification of novel genes that are potential targets of gamma irradiation. It will provide essential information on the immediate and long-term effects of gamma irradiation of breast cells that may be the key to further understanding of the mechanism of radiation-induced breast cancer.

NOVEL RADIATION RESPONSE GENES IDENTIFIED IN MCF10A CELLS

Jennifer L. Malone and Robert L. Ullrich
Department of Radiological and Environmental Health Sciences
Colorado State University

Introduction

Breast cancer may be induced with relatively high frequency by radiation. Ionizing radiation is one of the main treatment modalities used in the management of cancer. Radiation is used in the treatment of breast cancer, leukemia, and Hodgkin's lymphoma to kill cancerous cells. While the use of medical radiation has undoubtedly prolonged and saved the lives of many, it is not without side effects. A radiation dose-related increase in the incidence of breast cancer has been seen in women. When a woman receives significant radiation prior to the age of 20, she becomes more likely to develop breast cancer. Thus there is considerable interest in understanding the cellular response to DNA-damaging agents, particularly because the ability to deliver a curative dose of radiation is frequently limited by the adverse reaction of normal tissues within the radiation treatment field. One approach to this problem is to understand the molecular mechanisms underlying the radiation responses of normal tissue so that critical molecular pathways can be manipulated to improve the therapeutic ratio and hence, the chance of a cure.

We propose that the expression of several unknown genes is directly affected by gamma radiation. Abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We have established an assay that allows us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. Gene trapping is a form of insertional mutagenesis specifically designed to disrupt gene function by producing intragenic integration events. By employing a polyadenylation (poly A) trap a mRNA transcribed from a selectable marker gene lacking a poly A signal in a gene-trap vector is stabilized only when a gene-trap vector captures a poly A signal. Since poly A trapping occurs independently of the expression of the target genes, any gene could potentially be identified at almost equal probability regardless of the relative abundance of its transcripts in target cells. Upon treatment with ionizing radiation, the radiation-response genes identified will be sequenced through the rapid amplification of cDNA ends (RACE) procedure.

Hypothesis and Rationale

Mutation of novel genes or their abnormal expression in response to a single dose of gamma radiation is one of the causes of early breast carcinogenesis.

In experimental models, ionizing radiation induces mammary transformation both *in vivo* and *in vitro*, however, the cellular and molecular mechanisms of radiation-induced carcinogenesis are not known. To understand the mechanisms, it is necessary to determine the conditions that modulate the susceptibility of this target tissue to ionizing radiation. The assessment of the transforming ability of ionizing radiation on breast epithelium requires a highly proliferating cell population, which in turn has to express normal breast phenotypes. Since ionizing radiation induces features of neoplastic transformation in human breast cells, the identification of malignant phenotypes involved in breast cancer are of critical importance in understanding the pathogenesis of the disease.

The aim of our assay is to identify genes responsive to gamma irradiation through the use of gene trapping. Gene trapping will tag the radiation-responsive genes and we will be able to monitor their expression levels using GFP. At the same time, we will be introducing a single allele gene disruption into the radiation-responsive gene by the integration of our poly A trap vector which will decrease expression. We believe that by utilizing the gene-trapping technique some of the unknown genes that are contributing to familial and non-familial breast cancer can be identified. During radiation treatment of breast cancer, some of the cells surrounding a targeted tumor also become exposed, leading to mutations. It is of great importance that these radiation-induced mutations be identified so that a secondary cancer does not develop. The radiation-responsive genes identified can then serve as markers for screening and hopefully aid in early detection.

Methods

MCF-10A is a spontaneously non-tumorigenic immortalized human breast epithelial cell line. We have generated RET-infected G418-resistant MCF-10A clones, which essentially are a gene-trapped library of mammary epithelial cells. This library can be used in identifying genes that are activated or inhibited in mammary epithelial cells in response to different genotoxic agents or developmental signals. Since the infection rate is one virus per cell, this library represents cells in which one functional gene is disrupted by the integration of the vector. It provides the strong base for the identification of novel genes that may be involved in essential signaling pathways in human mammary epithelial cells.

We have established a detection assay using the reporter, Green Fluorescent Protein (GFP), that has been incorporated into the genome of the clones and whose expression is regulated by the endogenous promoters of the trapped genes. We will compare basal GFP expression before and after exposure to varying low dose gamma radiation (0-4 Gy) using replica plates of MCF10A clones. We will then identify the gene/s involved by using a polymerase chain reaction protocol (3' RACE) and sequencing analysis. Next, we will further characterize the clones that are affected by gamma irradiation by performing real-time PCR to analyze gene expression, soft agar assays to analyze anchorage-independent growth and tumorigenicity assays to confirm if the gene trap clones cause transformation.

Results

The gene-trapped MCF-10A clones were sorted by flow cytometry into GFP+ and GFP- expression levels. The GFP+ sort pool was then further sorted into GFP high, medium and low expression levels. Replica plates were made for both the GFP+ and GFP- sorts. The GFP expression level was measured using an ELISA assay both before and after a 2.0 Gy dose of IR.

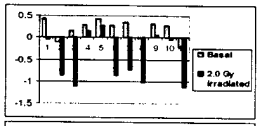


Figure 1. GFP negative sort population of MCF10A gene trapped single cell assay clones. A small minority of clones were upregulated, but most of the clones were downregulated.

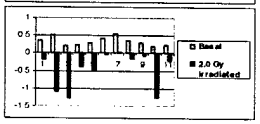


Figure 2. GFP positive sort population of MCF10A gene trapped single cell assay clones. Clones that were either up or down-regulated at least 2-fold in comparison to basal levels were expanded to determine what gene was trapped.

3' RACE analysis was implemented to determine the identity of the trapped genes in the MCF10A clones that were up- or down-regulated in response to a 2.0 Gy dose of ionizing radiation. The five genes identified through a BLAST homology search were:

Human DORA reverse strand protein 1 (DREV1) : **G10+**
Human Androgen Receptor : **G11-**
Human Eukaryotic Translation Elongation Factor 1 Beta 2 : **E8+**
Human Creatine Kinase Gene : **B5+**
Human Ribosomal Protein L27 : **A4-**

Quantitative real-time PCR analysis of mRNA expression of gene-trapped MCF10A clones irradiated with 2.0 Gy ionizing radiation and MCF10A epithelial cells harvested at various time points after 2.0 Gy dose. The following calculation was done to obtain the results presented in the graphs below:

$2^{-\Delta\Delta Ct}$

This formula calculates the relative gene expression by using the Ct values obtained from a PCR base line subtracted graph calculated by the iCycler software. The Ct values from the MCF10A gene trapped clones were compared to the endogenous control GAPDH to obtain the ΔΔCt values. MCF10A parental cells were used as a calibrator and set to 1.

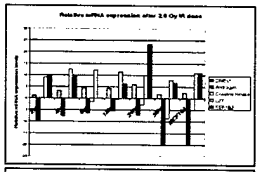


Figure 3. Real-Time PCR analysis of MCF10A gene-trapped IR clones. The relative mRNA expression levels of the IR gene-trapped clones to the parental MCF10A cell line at various time points following IR.

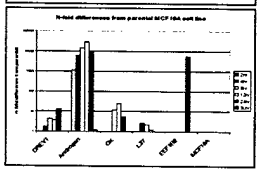


Figure 4. Real-Time PCR analysis of MCF10A gene-Trapped IR clones. The fold change in mRNA expression level of the IR gene-trapped clones compared to the parental line, MCF10A.

Cell cycle analysis was performed by flow cytometry to investigate if the DNA content varied during the phases of the cell cycle after a 2.0 Gy dose of ionizing radiation. Analysis was performed on the parental MCF10A cell line without IR and at varying time points after a 2.0 Gy dose of IR. This analysis was also performed on the five gene-trapped clones both with and without a 2.0 Gy dose of IR. This experiment was done to disregard any fluctuations in the mRNA expression levels being due to a cell cycle phenomenon.

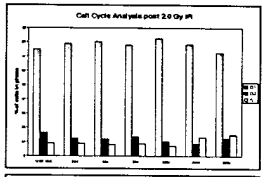


Figure 5. Cell cycle analysis of DNA content post IR. Analysis was performed after a 2.0 Gy dose of IR and analyzed at various time points post IR via flow cytometry. MCF10A parental cell line was used as a control.

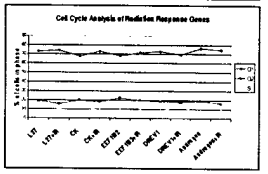


Figure 6. Cell cycle analysis of DNA content in potential radiation response gene-trapped clones. The DNA content in G1, G2, and S-phase was performed by flow cytometry analysis. The gene-trapped clones were analyzed with and without a 2.0 Gy dose of IR.

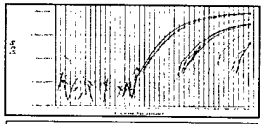


Figure 7. Amplification plot: Delta Rn vs. Cycle number. Real-time PCR experiment analyzing the mRNA expression levels of the five radiation response genes at various doses of IR.

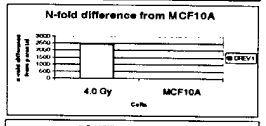


Figure 8. Real-Time PCR analysis of DREV1 mRNA expression. The N-fold difference in mRNA expression levels of DREV1 in MCF10A cells with either a dose of 4.0 Gy or without IR.

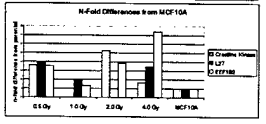


Figure 9. Real-Time PCR analysis of mRNA expression levels. The N-fold difference in mRNA expression levels of MCF10A at various doses of IR for the following genes: creatine kinase, ribosomal protein L27, and translation elongation factor 1 beta 2.

Conclusions

This study identified five genes that are potential targets of gamma irradiation. The immediate and long-term effects of gamma irradiation on breast cells may be the key to further understanding the mechanism of radiation-induced breast cancer. This assay will also be useful for testing other potential environmental risk factors involved in breast carcinogenesis that may prove to be useful as markers for early detection of breast cancer and targets for therapeutic intervention.

This work was supported by a grant from the Department of Defense Breast Cancer Research Program, DAMD17-02-1-0349 to J. Malone

Novel radiation response genes identified in MCF10A gene-trapped cells.

Jennifer Malone and Robert Ullrich
Department of Environmental and Radiological Health Sciences, Colorado State University

Abstract Category: Mutagenesis/Clastogenesis/Carcinogenesis

Objective/Hypothesis: In this study, we plan to establish an assay to identify novel genes that are affected by gamma irradiation and to characterize their function and role in early breast carcinogenesis. We hypothesize that the mutation of these genes or their abnormal expression in response to gamma irradiation is one of the causes of breast carcinogenesis.

Specific Aims: The specific aims of this study are:

1. To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones; 2. To determine the effect of gamma irradiation on the expression of the reporter, green fluorescent protein (GFP); 3. To characterize the effect of gamma irradiation on the transformation of human mammary epithelial cells; 4. To identify the trapped genes affected by gamma irradiation in breast epithelial cells.

Methods: We plan to establish an assay that will allow us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. We will be able to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Gene-trapped clones that are affected by radiation will be isolated and further analyzed to see if the varying radiation doses can lead to malignant transformation.

Results: The MCF10A gene-trapped library has been established. Basal GFP levels have been measured. Gamma irradiation of the single cell clones at both 1.0 and 2.0 Gy has been performed. Clones that were either up- or down-regulated at least 2-fold in response to the radiation treatment have been expanded for RACE and sequencing analysis. The genes identified through sequencing have been analyzed by real time PCR.

Study Design: Using the poly-A trap retrovirus RET, we have established a gene-trapped library of clones from human mammary epithelial cells (MCF10A). It provides the strong base for the identification of novel genes that may be involved in essential signaling pathways in human mammary epithelial cells. We propose to establish a detection assay using the reporter gene GFP that has been incorporated into the genome of the cells, whose expression is regulated by endogenous promoters of the trapped genes. We will compare basal GFP expression before and after exposure to varying low doses of gamma radiation (0-2 Gy) using replica plates of MCF10A gene-trapped clones. We will then identify the gene(s) involved by using a polymerase chain reaction protocol and sequencing analysis. Next, we will further characterize the clones that are affected by gamma irradiation by performing colony formation assays (to determine survival), anchorage-independent growth and tumorigenicity assays on transformed clones that grow in soft agar.

Conclusions: This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation-responsive genes that can lead to breast cancer as well as identify new markers for early detection of breast cancer. This study will focus on the identification of novel genes that are potential targets of gamma irradiation. It will provide essential information on the immediate and long-term effects of gamma irradiation of breast cells that may be the key to further understanding of the mechanism of radiation-induced breast cancer.

AACR Special Conference: Advances in Breast Cancer Research Abstract

Breast cancer may be induced with relatively high frequency by radiation. Ionizing radiation is one of the main treatment modalities used in the management of cancer. A radiation dose-related increase in the incidence of breast cancer has been seen in women. When a woman receives significant radiation prior to the age of 20, she becomes more likely to develop breast cancer. Thus there is considerable interest in understanding the cellular response to DNA-damaging agents, particularly because the ability to deliver a curative dose of radiation is frequently limited by the adverse reaction of normal tissues within the radiation treatment field. One approach to this problem is to understand the molecular mechanisms underlying the radiation responses of normal tissue so that critical molecular pathways can be manipulated to improve the therapeutic ratio and hence, the chance of a cure. We propose that the expression of several genes is directly affected by gamma radiation. Abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We plan to screen breast cells that contain a single gene mutation using a technique called gene trapping. We will be able to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation response genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Cells that are affected by radiation will be isolated and further analyzed to see if the changes can lead to the malignant transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation and genes that can cause breast cancer that are induced by radiation as well as identify markers for early detection of breast cancer and targets for therapeutic intervention.

IDENTIFICATION OF NOVEL GENES AFFECTED BY GAMMA IRRADIATION USING A GENE-TRAPPED LIBRARY OF HUMAN MAMMARY EPITHELIAL CELLS

Jennifer L. Malone and Robert L. Ullrich
Department of Radiological and Environmental Health Sciences
Colorado State University

Introduction

Breast cancer is one of the most common cancers among women and is the second leading cause of cancer death in women in the United States, exceeded only by lung cancer. There are both genetic and environmental components associated with breast cancer. Genetic risks factors include mutations in such genes as BRCA1, BRCA2, and ATM. Ionizing radiation (IR), such as gamma irradiation used for chest area radiation treatment, is a known risk factor that can cause breast cancer. Radiation therapy, used as part of breast conserving therapy for early breast cancer, is directed to normal breast tissue in order to eradicate remaining malignant cells by inducing DNA damage and cell death.

We propose that the expression of several unknown genes is directly affected by gamma radiation. Abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. We will be able to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Cells that are affected by radiation will be isolated and further analyzed to see if the changes can lead to the malignant transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation and genes that can cause breast cancer that are induced by radiation as well as identify markers for early detection of breast cancer and targets for therapeutic intervention.

Hypothesis and Rationale

Mutation of novel genes or their abnormal expression in response to a single dose of gamma radiation is one of the causes of early breast carcinogenesis.

In experimental models, ionizing radiation induces mammary transformation both *in vivo* and *in vitro*, however, the cellular and molecular mechanisms of radiation-induced carcinogenesis are not known. To understand the mechanisms, it is necessary to determine the conditions that modulate the susceptibility of this target tissue to ionizing radiation. The assessment of the transforming ability of ionizing radiation on breast epithelium requires a highly proliferating cell population, which in turn has to express normal breast phenotypes. Since ionizing radiation induces features of neoplastic transformation in human breast cells, the identification of malignant phenotypes involved in breast cancer are of critical importance in understanding the pathogenesis of the disease.

The aim of our assay is to identify genes responsive to gamma irradiation through the use of gene trapping. Gene trapping will tag the radiation-responsive genes and we will be able to monitor their expression levels using GFP. At the same time, we will be introducing a single allele gene disruption into the radiation-responsive gene by the integration of our poly A trap vector which will decrease expression. We believe that by utilizing the gene-trapping technique some of the unknown genes that are contributing to familial and non-familial breast cancer can be identified. During radiation treatment of breast cancer, some of the cells surrounding a targeted tumor also become exposed, leading to mutations. It is of great importance that these radiation-induced mutations be identified so that a secondary cancer does not develop. The radiation-responsive genes identified can then serve as markers for screening and hopefully aid in early detection.

Methods

MCF-10A is a spontaneously non-tumorigenic immortalized human breast epithelial cell line. We have already generated RET-infected G418-resistant MCF-10A clones, which essentially are a gene-trapped library of mammary epithelial cells. At this point, this library can be used in identifying genes that are activated or inhibited in mammary epithelial cells in response to different genotoxic agents or developmental signals. Since the infection rate is one virus per cell, this library represents cells in which one functional gene is disrupted by the integration of the vector. It provides the strong base for the identification of novel genes that may be involved in essential signaling pathways in human mammary epithelial cells.

We have established a detection assay using the reporter gene GFP (green fluorescent protein) that has been incorporated into the genome of the cells, whose expression is regulated by endogenous promoters of the trapped genes. We will compare basal GFP expression before and after exposure to varying low dose gamma radiation (0-2 Gy) using replica plates of MCF10A clones. We will then identify the gene/s involved by using a polymerase chain reaction protocol (3' RACE) and sequencing analysis. Next, we will further characterize the clones that are affected by gamma irradiation by performing real-time PCR to analyze gene expression, soft agar assays to analyze anchorage-independent growth and tumorigenicity assays to confirm if the gene trap clones cause transformation.

Results

The gene-trapped MCF-10A clones were sorted by flow cytometry into GFP+ and GFP- expression levels, as seen in figure 1. The GFP+ sort pool was then further sorted into GFP high, medium and low expression levels as seen in figure 2.

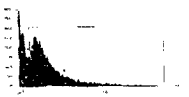


Figure 1. Initial sort of gene-trapped MCF-10A clones into GFP+ and GFP- pools.
Sort gate 1 is the GFP- pool and sort gate 2 is the GFP+ pool.

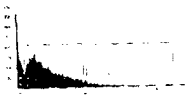


Figure 2. Cell sort of gene-trapped MCF-10A clones into high, medium and low GFP expression levels.
Sort gate 1 is the GFP medium pool and sort gate 2 is the GFP high pool.

Gene-trapped MCF10A-1023 cells were plated in 96-well plates to be replica plated in order to obtain single cell clones, not pooled clones. GFP expression was determined by a sandwich ELISA protocol and measured with a microplate reader. The GFP expression was visualized under a fluorescence microscope to verify the expression levels from the endogenous promoter of the trapped gene as either high, medium or low. As you can see below in figure 3, high, medium and low GFP expression was observed.

Figure 3. GFP expression of replica plated gene-trapped MCF-10A clones.
A. High GFP expressing M1023 gene-trapped clone from replica plate.
B. Medium GFP expressing M1023 gene-trapped clone from replica plate.
C. Low GFP expressing M1023 clone from replica plate.

Analysis of GFP expression data from 2.0 Gy irradiated single cell MCF10A gene trapped clones by microplate reader.

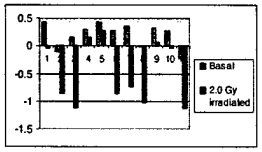


Figure 4. GFP negative sort population of MCF10A gene trapped single cell assay clones.
Some clones were upregulated, but most of the clones were downregulated. Clones that were either up or down-regulated at least 2-fold in comparison to basal levels were expanded to determine what gene was trapped.

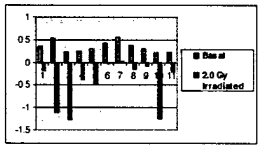


Figure 5. GFP positive sort population of MCF10A gene trapped single cell assay clones.

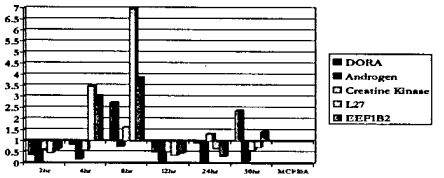
3' RACE analysis was implemented to determine the identity of the trapped genes in the MCF10A clones that were up- or down-regulated in response to a 2.0 Gy dose of ionizing radiation. The six genes identified through a BLAST homology search were:

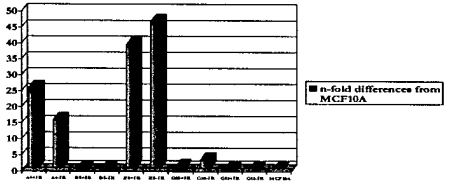
Human DORA reverse strand protein 1 (DREV1) : **G10+**
Human Androgen Receptor : **G11-**
Human Eukaryotic Translation Elongation Factor 1 Beta 2 : **E8+**
Human Creatine Kinase Gene : **B5+**
Human Ribosomal Protein L27 : **A4-**
Human DNA sequence from clone RP1290F20 on Chromosome 20 : **H4**

Quantitative real-time PCR analysis of mRNA expression of gene-trapped MCF10A clones irradiated with 2.0 Gy ionizing radiation and MCF10A epithelial cells harvested at various time points after 2.0 Gy dose. The following calculation was done to obtain the results presented in the graphs below:

$2^{-\Delta\Delta Ct}$

This formula calculates the relative gene expression by using the Ct values obtained from a PCR base line subtracted graph calculated by the iCycler software. The Ct values from the MCF10A gene trapped clones were compared to the endogenous control GAPDH to obtain the ΔΔCt values. MCF10A parental cells were used as a calibrator and set to 1.





Conclusions

This study identified six genes that are potential targets of gamma irradiation. The immediate and long-term effects of gamma irradiation on breast cells may be the key to further understanding the mechanism of radiation-induced breast cancer. Establishment of this assay will also be useful for testing other potential environmental risk factors involved in breast carcinogenesis that may prove to be useful as markers for early detection of breast cancer and targets for therapeutic intervention.

This work was supported by a grant from the Department of Defense Breast Cancer Research Program, DAMD17-02-1-0349 to J. Malone

Real Time PCR analysis of gene-trapped MCF10A clones

Jennifer Malone
Colorado State University
October 23, 2003

Research Overview

- Hypothesis: *Mutation of novel genes or their abnormal expression in response to a single dose of gamma radiation is one of the causes of early breast carcinogenesis.*
- **Specific aim 1.** To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF-10A clones.
- **Specific aim 2.** To determine the effect of gamma irradiation on expression of reporter protein GFP.
- **Specific aim 3.** To identify the "trapped" genes affected by gamma irradiation.
- **Specific aim 4.** To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells.

Breast Cancer & Radiation

- Breast cancer may be induced with relatively high frequency by radiation.
- Ionizing radiation is one of the main treatment modalities used in the management of cancer.
- Radiation is used in the treatment of breast cancer, leukemia, and Hodgkin's lymphoma to kill cancerous cells.
- A radiation dose related increase in the incidence of breast cancer has been seen in women.
- When a woman receives significant radiation prior to the age of 20, she becomes more likely to develop breast cancer.

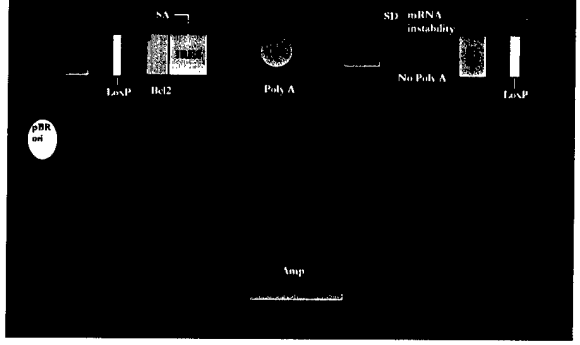
Gene Trapping

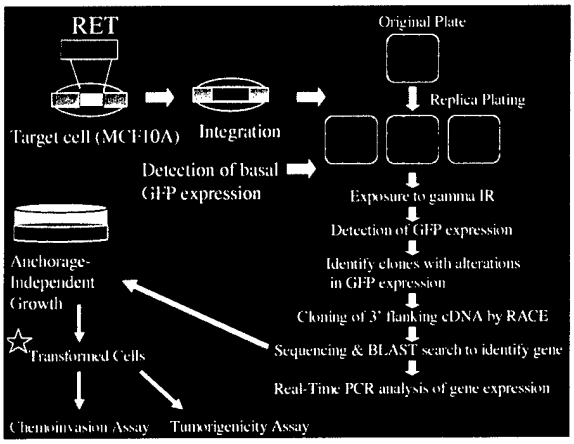
- Form of insertional mutagenesis.
- Disrupt gene function by intragenic integration.
- mRNA transcribed from a selectable marker gene lacking a poly A signal in a gene-trap vector is stabilized only when the gene trap vector captures a cellular poly A signal.
- Poly A trapping occurs independently of the expression of target genes, regardless of its expression.
- The sequence of the 'trapped' gene can be identified using techniques that are based on the polymerase chain reaction (PCR), and this can lead to the isolation of novel genes regardless of their level of expression in vivo.

Materials

- MCF10A: immortalized human mammary epithelial cells that arose spontaneously.
- pRET: retroviral vector used for gene trapping. Contains a very strong splice acceptor and a poly A signal used to disrupt the trapped gene. Neo marker to select clones with integration and GFP for monitoring of endogenous trapped gene's expression level.

Map of pRET





Sequencing Results

- 31 irradiated gene trapped clones sequenced
- Sequencing results plugged into BLAST
- 6 clones were homologous to known gene sequences:
  - Human DORA reverse strand protein 1 (DREV1) : **G10+**
  - Human Androgen Receptor : **G11-**
  - Human Eukaryotic Translation Elongation Factor 1 Beta 2 : **E8+**
  - Human Creatine Kinase Gene : **B5+**
  - Human Ribosomal Protein L27 : **A4-**
  - Human DNA sequence from clone RP1290E20 on Chromosome 20 : **H4+**

The Next Step

- The six clones that had yielded homologous genes through BLAST search were analyzed.
- The clones were grown up and the cells were harvested for RNA extraction.
- RT PCR was performed and the cDNA was used in a real time PCR reaction.

Reverse Transcription

- 2.0 Gy irradiated gene trapped clones that had yielded homologous BLAST results were RT PCR to analyze gene expression of the selected genes of interest
- Conditions:

  Incubation: 25°C for 10 minutes

  Reverse Transcription: 48°C for 30 minutes

  RT Inactivation: 95°C for 5 minutes

Real Time PCR

- Conditions:

  UNG Incubation: 50 C for 2 minutes

  AmpliTaq Gold Activation: 95 C for 10 minutes

  PCR: started out at 40 cycles and increased up to 55

  Denature: 95 C for 15 seconds

  Anneal, Extend: 60 C for 1 minute

Primers & Probes

- Three forward & reverse primers were designed by Primer Express for each gene of interest
- TaqMan probes were designed by Primer Express for each gene of interest
- **Primer design requirements**:

  The T_m should be 58 to 60°C

  Keep G-C content in 20-80% range

  The five nucleotides at the 3' end should have no more than two G/C bases

  Forward & reverse primers should be as close as possible to the probe w/o overlapping it

  Avoid runs of an identical nucleotide, especially G
- **Probe design requirements**:

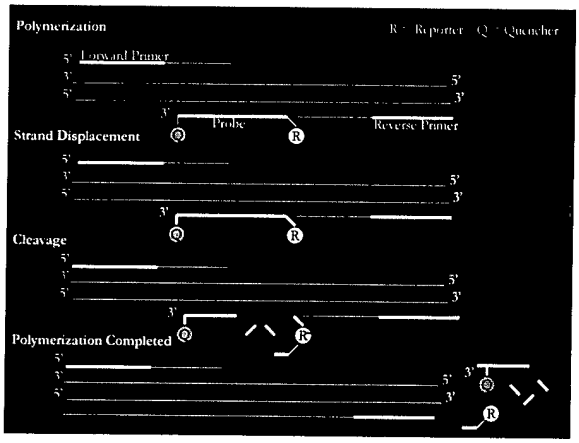
  Avoid runs of an identical nucleotide, especially G

  The 5' end of the probe cannot be a guanosine residue

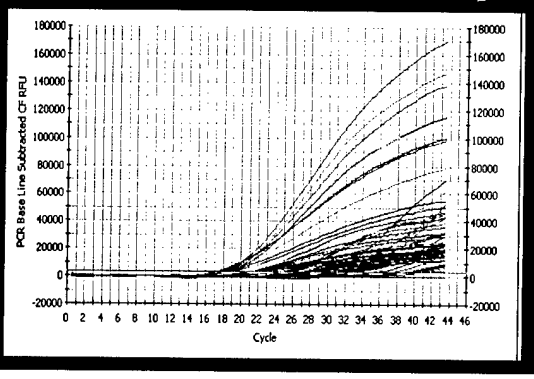
  The T_m should be 65 to 67°C

TaqMan Probes

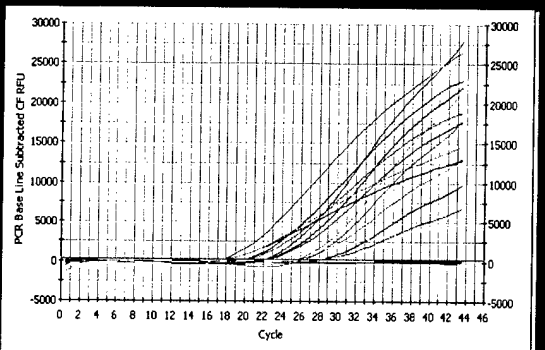
- The reporter dye FAM was used to label the 5' end of my gene specific probes
- The chromophore TAMRA was used to quench the probe on the 3' end



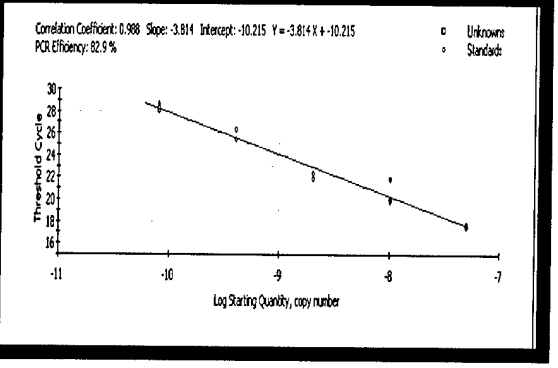
PCR Base Line Subtracted Graph



GAPDH Endogenous Control Graph



GAPDH Standard Curve Graph



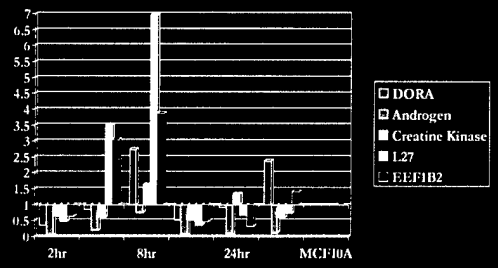
Data Calculations

- Absolute Standard Method for data quantification:

  $\Delta CT = CT\ (target) - CT\ (GAPDH)$
- Comparative expression level for data quantification:

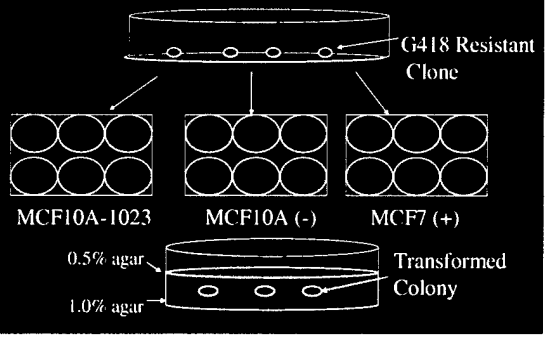
  $= 2^{-\Delta\Delta CT}$

Relative Gene Expression of MCF10A cells collected post 2.0 Gy IR



Relative Gene Expression of Irradiated Gene-Trapped MCF10A Clones

Summary of Soft Agar Assay



Future Directions

- Analyze the cell cycle distribution for my gene trapped irradiated clones and MCF10A various time points after IR by flow cytometry
- To determine if anchorage-independent clones are fully malignant, MCF10A gene trapped clones will be injected subcutaneously into the subscapular area of 3-week old irradiated athymic female nude mice (BALB/c background).
- Characterize what other known proteins the radiation responsive gene(s) identified bind to

Acknowledgements

- Dr. Robert Ullrich
- All members of the Ullrich lab
- My committee members
- Thanks!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!